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(54) Title: TREATING DISORDERS BY APPLICATION OF INSULIN-LIKE GROWTH FACTORS AND ANALOGS

(57) Abstract

A method of enhancing the survival of neuronal cells in a mammal, the cells being at risk of dying, the method comprising administering to the mammal an effective amount of at least one of the following substances: IGF-I; a functional derivative of IGF-I; IGF-II; a functional derivative of IGF-II; IGF-III; or a functional derivative of IGF-III.

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TREATING DISORDERS BY APPLICATION OF INSULIN-LIKE GROWTH FACTORS AND ANALOGS

Background of the Invention

The present invention relates to therapeutic polypeptides useful, e.g., for the treatment of neurological and other disorders.

Insulin-like growth factors (IGFs) have been identified in various animal species as polypeptides that act to stimulate growth of cells in a variety of tissues (see Baxter et al., Comp. Biochem. Physiol. 91B:229-235 (1988); and Daughaday et al., Endocrine Rev. 10:68-91 (1989) for reviews), particularly during development (see D'Ercole, J. Devel. Physiol. 9:481-495 (1987) for review). The IGFs, each of which has a molecular weight of about 7,500 daltons, are chemically related to human proinsulin: i.e. they possess A and B domains that (1) are highly homologous to the corresponding domains of proinsulin, and (2) are connected by a smaller and unrelated C domain. A carboxyl-terminal extension, the D domain, is also present in IGFs but is not found in proinsulin.

Certain polypeptide fragments of the IGFs have proven to be useful as antigens to raise antibodies specific for each of the IGFs (see, e.g., Japanese Patent Application No. 59065058; Hintz and Liu, J. Clin. Endocr. Metab. 54:442-446 (1982); Hintz et al., Horm. Metab. Res. 20:344-347 (1988)). Using labelled IGF-specific antibodies as a probe, IGF-I and IGF-II (sometimes respectively termed "somatomedin C" and "somatomedin A") have been found in a variety of tissues, including the mammalian central nervous system(CNS); the presence in the CNS of mRNAs encoding these polypeptides suggests local synthesis in the CNS (see Baskin

et al., TINS 11:107-111 (1988) for review). In addition, IGF-III (or "brain IGF"), a truncated form of IGF-I lacking the latter protein's three N-terminal amino acid residues, has been found in fetal and adult human brain (Sara et al., Proc. Natl. Acad. Sci. USA 83:4904-4907 (1986), as well as in colostrum (Francis et al., Biochem. J. 251:95-103 (1988)). Two different IGF receptors have been identified in the adult human CNS (Baskin et al., 1988, *supra*), including in the brain (Sara et al., Neurosci. Let. 34:39-44 (1982)). In addition, European Patent No. 227,619 describes evidence for a third type of IGF receptor located in human fetal membranes. Complicating research in this area are (1) evidence that the insulin receptor of brain membranes recognizes not only insulin but also the IGFs; (2) the finding that one of the two types of adult IGF receptors exhibits some affinity for insulin as well as for both IGF-I and II, and (3) current uncertainty as to the physiological significance of binding of IGF-II to the second type of adult IGF receptor (Baskin et al., 1988, *supra*).

IGF-I and IGF-II appear to exert a stimulatory effect on development or proliferation of a wide range of susceptible cell types (see Daughaday et al., 1989, *supra*, for review). Treatment with the IGFs or with certain polypeptide fragments thereof has been variously suggested as a bone repair and replacement therapy (European Patent Application No. 289 314), as a means to counteract certain harmful side effects of carcinostatic drugs (Japanese Patent No. 63196524), and as a way to increase lactation and meat production in cattle and other farm animals (Larsen et al., U.S. Patent No. 4,783,524). Each of the IGFs also appears to enhance the survival, proliferation and/or neurite outgrowth of cultured embryonic neurons (which, unlike mature neurons, have not yet lost their ability to undergo

cell division) from various parts of the CNS (Aizenman et al., Brain Res. 406:32-42 (1987); Fellows et al., Soc. Neurosci. Abstr. 13:1615 (1987); Onifer et al., Soc. Neurosci. Abstr. 13:1615 (1987); European Patent No. 227,619 and from the peripheral nervous system (Bothwell, J. Neurosci Res. 8:225-231 (1982); Recio-Pinto et al., J. Neurosci 6:1211-1219 (1986)). In addition, the IGFs have been shown to affect the development of undifferentiated neural cells: human neuroblastoma tumor cells were shown to respond to added IGFs by extending neurites (Recio-Pinto and Ishii, J. Neurosci. Res. 19:312-320 (1988)) as well as by undergoing mitosis (Mattson et al., J. Cell Biol. 102:1949-54 (1986). As the induction of the enzyme ornithine decarboxylase has been shown to correlate with the stimulation of mitotic activity of these cells, an assay for cell proliferation has been developed based upon measuring the level of activity of this enzyme (Mattsson et al., 1986).

Developing forebrain cholinergic neurons (cultured rat septal neurons) are sensitive to a variety of growth factors _in vitro_. Addition of nerve growth factor (NGF) to the culture medium increases the number of cells positive for the expression of transmitter-specific enzymes (acetyl choline esterase (AChE) and choline acetyl transferase (ChAT)) (Hartikka and Hefti, J. Neuroscience 8:2967-2985 (1988). Thyroid hormone also increases the level of ChAT in cultured septal neurons and thyroid hormone in combination with NGF results in a stimulation of ChAT activity much greater than the sum of the effects of individual addition of these two substances (Hayashi and Patel, Dev. Brain Res. 36:109-120 (1987)). IGF-I, IGF-II, and insulin also induce ChAT activity in cultured septal neurons (Knusel et al., J. of Neuroscience 10:558-570 (1990)). When NGF and

insulin are both added to the culture medium the effect on ChAT activity is additive, but the effects of IGF-I or IGF-II in combination with insulin are not additive (Knusel et al., 1990, *supra*).

In vivo studies also support the hypothesis that the IGFs play a role in development and differentiation of the immature peripheral and central nervous systems (Sara et al., J. Dev. Physiol. 1:343-350 (1979); Philipps et al., Pediatr. Res. 23:298-305 (1988); Sara et al., Prog. Brain Res. 73:87-99 (1988)), although the physiological nature of this role remains uncertain. Once the neuronal cells of the CNS reach maturity, they do not undergo further cell division.

Neurotrophic factors other than the IGFs have been proposed as a potential means of enhancing neuronal survival, for example as a treatment for the neurodegenerative diseases amyotrophic lateral sclerosis (using skeletal muscle-derived proteins having apparent molecular weights in the 20,000-22,000 dalton and 16,000-18,000 dalton ranges: PCT Application No. PCT/US88/01393), and Alzheimer's disease (using phosphoethanolamine: PCT Application No. PCT/US88/01693). Sara et al., although finding a "significant elevation" in serum and cerebrospinal fluid somatomedin (IGF) levels in patients suffering from Alzheimer's disease compared to normal controls, nevertheless conclude:

> Whether somatomedins play a casual (sic) role in the etiology of the dementia disorders of the Alzheimer type remains to be determined. However, since somatomedins stimulate the uptake of amino acids into brain tissue, their administration may provide beneficial therapeutic effects. Finally, the fall in somatomedins observed in normal elderly patients raises the general question of

> their role in cell aging. (citation omitted; Sara et al., Neurobiol. Aging 3:117-120, 119 (1982)).

In a report that IGF-I, but not IGF-II, stimulates the immediate (i.e. within 20 min.) release of acetylcholine from slices of adult rat brain, a process thought to be related to transitorily increased neurotransmission of acetylcholine rather than to increased cholinergic enzyme activity, Nilsson et al., Neurosci. Let. 88:221-226, 221, 224 (1988), point out that

> [One] of the major deficits in Alzheimer's disease concerns the cholinergic system of the brain, where a reduced synthesis and release of [acetylcholine] has been found....It is of considerable importance to further investigate the role of IGFs in neurodegenerative disorders such as Alzheimer's disease... (citations omitted).

Using antibody specific for IGF-I to detect an increase in the presence of IGF-I in injured peripheral nerves, notably in the non-neuronal cells named "Schwann cells", Hansson et al., Acta Physiol. Scand. 132:35-41, 38, 40 (1988), suggest that

> Thus, increased IGF-I immunoreactivity is observed in regenerating peripheral nerves after any injury and seems to form part of a general reaction pattern, most evident in the Schwann cells. Our ultrastructural studies have revealed that the Schwann cells undergo hypertrophy after vibration trauma, and show signs of activation, i.e. the granular endoplasmic reticulum and Golgi complex increased in extent. We thus interpret the increase in IGF-I immunoreactivity in the Schwann cells, documented in this study on vibration-exposed nerves, as part of a transient, reactive response beneficial for the early stages of repair processes....We consider the increase in IGF-I immunoreactivity to

> reflect mainly the initial reactions in a chain of events resulting in repair of the injured tissue or organ [although this increase] may be interpreted to reflect disturbed axoplasmic transport [of IGF-I molecules], due in part to the diminution of microtubules reported to occur after vibration exposure. (citation omitted)

Further, Sjoberg et al., Brain Res. 485:102-108 (1989), have found that local administration of IGF-I to an injured peripheral nerve stimulates regeneration of the nerve as well as proliferation of associated non-neuronal cells.

Several methods have been employed to decrease the susceptibility of polypeptides to degradation by peptidases, including, e.g., substitution of D-isomers for the naturally-occurring L-amino acid residues in the polypeptide (Coy et al., Biochem. Biophys. Res. Commun. 73:632-8 (1976)). Where the polypeptide is intended for use as a therapeutic for disorders of the CNS, an additional problem must be addressed: overcoming the so-called "blood-brain barrier," the brain capillary wall structure that effectively screens out all but selected categories of molecules present in the blood, preventing their passage into the brain. While the blood-brain barrier may be effectively bypassed by direct infusion of the polypeptide into the brain, the search for a more practical method has focused on enhancing transport of the polypeptide of interest across the blood-brain barrier, such as by making the polypeptide more lipophilic, by conjugating the polypeptide of interest to a molecule which is naturally transported across the barrier, or by reducing the overall length of the polypeptide chain (Pardridge, Endocrine Reviews 7:314-330 (1986); U.S. Patent No. 4,801,575.

Summary of the Invention

In general, the invention features a method of enhancing the survival of neuronal cells at risk of death, preferably non-mitotic neuronal cells and/or cholinergic neuronal cells, in a mammal, preferably in the context of a therapeutic treatment of neuronal tissues which are suffering from the effects of aging, of injury, or of a disease e.g., Alzheimer's disease, stroke, epilepsy, amyotrophic lateral sclerosis, or Parkinson's disease, by administering to the mammal an effective amount of at least one of the following: IGF-I, a functional derivative of IGF-I, IGF-II, or a functional derivative of IGF-II, IGF-III, or a functional derivative of IGF-III, with or without the administration of an effective amount of NGF, ciliary neurotrophic factor (CNTF), or a functional derivative thereof.

The invention also features a method of enhancing the survival of neuronal cells at risk of death, preferably non-mitotic neuronal cells and/or cholinergic neuronal cells, in a mammal, preferably in the context of a therapeutic treatment of neuronal tissues which are suffering from the effects of aging, of injury, or of a disease, e.g., Alzheimer's disease, stroke, epilepsy, amyotrophic lateral sclerosis, or Parkinson's disease, by treating said mammal with a first treatment including administration of a cell survival promoting amount of a growth factor, e.g., IGF-I, IGF-II, or IGF-III, or a functional derivative of the growth factor (e.g., a fragment, analog, or analog of a fragment of the first growth factor), alone, or in a biologically active combination with another such growth factor or functional derivative, and then treating said mammal with a second treatment including administration of a nerve transmitter

increasing amount of a transmitter enhancer e.g., NGF, CNTF, or a functional derivative of the transmitter enhancer (e.g., a fragment, analog, or analog of a fragment of the transmitter enhancer). In preferred embodiments, fragments, analogs, or analogs of fragments of IGF-I, IGF-II, IGF-III, or NGF are administered.

The invention also features a method of enhancing the cholinergic activity (i.e., acetylcholine-synthesizing capacity) of cholinergic neuronal cells in a mammmal, preferably non-mitotic neuronal cells, and preferably in the context of a therapeutic treatment of neuronal tissues which are suffering from the effects of aging, of injury, or of a disease, e.g., Alzheimer's disease, stroke, epilepsy, amyotrophic lateral sclerosis, or Parkinson's disease, by administering to the mammal an effective amount of one or more of the following: IGF-I, IGF-II, IGF-III, a functional derivative of IGF-I, or a functional derivative of IGF-II or a functional derivative of IGF-III (preferably administering a fragment of IGF-I, IGF-II, or IGF-III, or, alternatively, administering an analog of IGF-I, of IGF-II, or an analog of a fragment of IGF-I or IGF-II), with or without the administration of an effective amount of NGF, CNTF, or a functional derivative thereof, provided that if IGF-I or IGF-II is administered, NGF or a functional derivative thereof is also administered.

The invention also features a method of enhancing the cholinergic activity (i.e., acetylcholine-synthesizing capacity) of cholinergic neuronal cells in a mammmal, preferably non-mitotic neuronal cells, and preferably in the context of a therapeutic treatment of neuronal tissues which are suffering from the effects of aging, of injury, or of a disease, e.g., Alzheimer's disease, stroke, epilepsy, amyotrophic lateral sclerosis, or Parkinson's disease, by

treating said mammal with a first treatment including administration of a cell survival promoting amount of a growth factor, e.g., IGF-I, IGF-II, or IGF-III, or a functional derivative of the growth factor (e.g., a fragment, analog, or analog of a fragment), alone, or in a biologically active combination with another such growth factor or functional derivative, and then treating said mammal with a second treatment including an administration of a nerve transmitter increasing amount of a transmitter enhancer, e.g., a factor that increases the level of a transmitter specific enzyme in the cell, e.g., NGF, CNTF, or a functional derivative of a transmitter enhancer (e.g., a fragment, analog, or analog of a fragment).

Another method of the invention features treating a head or spinal cord injury of a mammal, or a disease condition of a mammal, e.g., stroke, epilepsy, age-related neuronal loss, amyotrophic lateral sclerosis, Alzheimer's disease, or Parkinson's disease, by (1), administering to the mammal an effective amount of at least one of the following substances: IGF-I, a functional derivative of IGF-I, IGF-II, a functional derivative of IGF-II, IGF-III, or a functional derivative of IGF-III, with or without the administration of NGF, CNTF, or a functional derivative thereof, or by (2), treating said mammal with a first treatment including administration of a cell survival promoting amount of one or more of a first group of substances, e.g., IGF-I, a functional derivative of IGF-I, IGF-II, a functional derivative of IGF-II, IGF-III, or a functional derivative of IGF-III, and then treating said mammal with a second treatment including administration of a nerve transmitter increasing amount of a transmitter enhancer or a functional derivative thereof, e.g., NGF, CNTF, or a functional derivative thereof.

A particular advantage of the use of combined treatments as described above is the ability to reduce the required dose of one component, e.g., CNTF, which alone in a higher dose may exhibit unwanted side effects, i.e., toxicity.

The invention also features a method of modifying a ligand, preferably a neuroactive polypeptide, capable of binding to a receptor located on a cell surface, by first binding the ligand to a preparation of said receptor, then performing the modification procedure (preferably cationization, glycosylation, or increasing the lipophilicity of the polypeptide), and then releasing the modified ligand from the receptor.

The invention also features a method of enhancing neurite regeneration in a mammal, the method involving treating the mammal with a first treatment involving administration of a neurite regenerating amount of a growth factor, or a functional derivative thereof, and then treating the mammal with a second treatment involving administration of a nerve transmitter increasing amount of a transmitter enhancer, or a functional derivative thereof. The functional derivative of the growth factor may preferably be IGF-II(54-67)(SEQ ID NO:3), IGF-II(58-67)(SEQ ID NO:2), TYCAPAKSE (SEQ ID NO:1), IGF-I(55-70)(SEQ ID NO:4), or an analog of the growth factor, or an analog of a fragment of the growth factor, more preferably IGF-II(54-67;D-Y)(SEQ ID NO:45), or IGF-II(58-67; D-Y)(SEQ ID NO:46), or any of the other peptides listed herein.

Polypeptides administered in methods of the invention may be chemically modified in such a way as to increase the transport of the polypeptide across the blood-brain barrier, e.g., by modifications of the polypeptide

that increase lipophilicity, alter glycosylation, or increase net positive charge.

Embodiments of the invention include the administration of more than one neuroactive polypeptide. In preferred embodiments the combined desired effect of administration of the polypeptides is additive, and in more preferred embodiments the effect is synergistic.

In other preferred embodiments, where a fragment of IGF-II is administered, preferred IGF-II fragments include, but are not limited to, IGF-II(54-67)(SEQ ID NO:3), IGF-II(58-67)(SEQ ID NO:2), or may include analogs of IGF-II fragments, e.g., TYCAPAKSE (SEQ ID NO:1), IGF-II(54-67; D-Y) (SEQ ID NO:45), or IGF-II(58-67; D-Y) (SEQ ID NO:46). Where a fragment of IGF-I or IGF-III is administered, preferred IGF-I,III fragments may include IGF-I(55-70) (SEQ ID NO:4).

The invention also features a composition including a first component taken from the group of purified IGF-I, a purified functional derivative of IGF-I, purified IGF-II, a purified functional derivative of IGF-II, purified IGF-III, or a purified functional derivative of IGF-III, and a second component taken from the group of purified NGF, or a purified functional derivative of NGF. Purified means that the substance is of 95% or greater (by weight) purity, i.e., that it is substantially free of proteins, lipids, and carbohydrates with which it is naturally associated.

In another aspect, the invention includes a substantially pure peptide, wherein the peptide includes a sequence selected from the group consisting of the amino acid sequence TYCATPAK (SEQ ID NO:51), LETYCATP (SEQ ID NO:52), CATPAKSE (SEQ ID NO:53), YCAPAKSE (SEQ ID NO:54), YCAPA (SEQ ID NO:55), TYCAPA (SEQ ID NO:56), CAPAKSE (SEQ ID NO:24), EALLETYCATPAKSE (SEQ ID NO:36), ALLEKYCAKPAKSE (SEQ ID NO:37), and APSTCEYKA (SEQ ID NO:38). As a preferred

embodiment, these peptides can be used in any of the various methods of the invention.

The invention also includes the substantially pure peptides TYCAPAKSE (SEQ ID NO:1), TDYCAPAKSE (SEQ ID NO:50)[1], IGF-II(54-67)(SEQ ID NO:3), IGF-II(58-67)(SEQ ID NO:2), IGF-I(55-70)(SEQ ID NO:4), EPYCAPPAKSE (SEQ ID NO:5), or analogs of the above peptides, preferably wherein tyrosine-59 is a D-isomer of tyrosine, e.g., IGF-II(54-67; D-Y) (SEQ ID NO:45) or IGF-II(58-67; D-Y) (SEQ ID NO:46). Where a fragment of IGF-I or IGF-III is administered, preferred IGF-I and IGF-III fragments may include IGF-I(55-70) (SEQ ID NO:4).

The invention also includes a cyclic peptide, preferably of 5-40 amino acids, and most preferably of 6-25 amino acids. Preferably the cyclic peptide includes a fragment of the respective IGF-I, IGF-II, or IGF-III as at least a portion of its amino acid sequence. The cyclic peptide can include a disulfide bond between two cysteines of the peptide, the cysteines being located at either terminal or internal positions of the peptide. Alternatively or in addition to the disulfide bond, the cyclic peptide may include an amide bond between the amino and carboxyl ends of the peptide. Preferred cyclic peptides include, but are not limited to, those derived by cyclization, e.g., by disulfide bond formation or by amide bond formation, of the following peptides: CALLETYCATPAKSEC (SEQ ID NO:6), CTYCATPAKSEC (SEQ ID NO:7), CEPYCAPPAKSEC (SEQ ID NO:8), CTYCAPAKSEC (SEQ ID NO:9), CALLETDYCATPAKSEC (SEQ ID NO:47), CTDYCATPAKSEC (SEQ ID NO:48), CTDYCAPAKSEC (SEQ ID NO:49), CTYTAPAKSEC (SEQ ID NO:10), CALLETYATPAKSEC

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[1] DY and D-Y, as used herein, refer to the D-isomer of Tyrosine.

(SEQ ID NO:11), CRRLEMYCAPLKPAKSAC (SEQ ID NO:12), CGYGSSSRRAPQTC (SEQ ID NO:13), CYFNKPTGYGC (SEQ ID NO:14), CYFNKPTGYGSSSRRAPQTC (SEQ ID NO:15), CKPTGYGSSSRC (SEQ ID NO:16), the amino acid sequence CGCELVDALQFVC (SEQ ID NO:18), the amino acid sequence CDLRRLEMYCCPLKPAKSE (SEQ ID NO:21), CGPETLC (SEQ ID NO:26), CGYGSSSRRCPQTGIVDEC (SEQ ID NO:27), CGDRGFYFNKPTC (SEQ ID NO:28), CCPLKPAKSAC (SEQ ID NO:29), CDLRRLEMYAPLKPAKSAC (SEQ ID NO:30), the amino acid sequence CDLCLLETYC (SEQ ID NO:33), the amino acid sequence CDLCLLETYCATPAKSE (SEQ ID NO:35), CCYRPSETLC (SEQ ID NO:40), CRPCSRVSRRSRGIVEEC (SEQ ID NO:41), CGDRGFYFSRPC (SEQ ID NO:42), CCTPAKSEC (SEQ ID NO:43), and CDLCLLETATPAKSEC (SEQ ID NO:44). Amino acid residues of the cyclic peptides can be in the form of either L-amino acids, or in the form of an amino acid analog listed in Table 2, e.g., D-amino acids. The residues flanking the amino acid sequence are preferably homologous to the naturally occurring sequence of IGF-I or to the naturally occurring sequence of IGF-II.

The invention also includes a substantially pure peptide, wherein the peptide is selected from the group consisting of the amino acid sequence CDLRRLEMYC (SEQ ID NO:19), the amino acid sequence CCFRSCDLRRLEMYC (SEQ ID NO:20), the amino acid sequence CCFRSC (SEQ ID NO:22), and the amino acid sequence CFRSC (SEQ ID NO:23), wherein the peptide is cyclized by a covalent bond between two residues of the peptide.

The invention also includes a substantially pure peptide, wherein the peptide is selected from the group consisting of the amino acid sequence CGGELVDTLQFVC (SEQ ID NO:32), the amino acid sequence CCFRSCDDLALLETYC (SEQ ID NO:34), wherein the peptide is cyclized by a covalent bond between two residues of the peptide.

The invention also includes a substantially pure cyclized peptide consisting essentially of the amino acid sequences CGCELVDALQFVC (SEQ ID NO:18) and CCFRSCDLRRLEMYC (SEQ ID NO:20), wherein the cyclized peptide includes at least one covalent bond between two residues of the looped peptide.

The invention also includes a substantially pure cyclized peptide consisting essentially of the amino acid sequences CGGELVDTLQFVC (SEQ ID NO:32) and CCFRSCDLCLLETYC (SEQ ID NO:39), wherein the cyclized peptide includes at least one covalent bond between two residues of the cyclized peptide.

As a preferred embodiment to any of the various methods of the invention, the functional derivative is a retro-inverso peptide, preferably a retro-inverso peptide that is homologous to IGF-I, or a fragment thereof, or a retro-inverso peptide that is homologous to IGF-II, or a fragment thereof. A "retro-inverso peptide", as used herein, refers to a peptide with a reversal of the direction of the peptide bond at at least one position, i.e., a reversal of the amino- and carboxy- termini with respect to the side chain of the amino acid. Retro-inverso peptides may contain L-amino acids or D-amino acids, or a mixture of L-amino acids and D-amino acids.

With respect to any of the IGF-I or IGF-II peptides listed herein, most preferred are linear and cyclic peptides that contain at least one cysteine residue that is not involved in disulphide bond formation. In some cases where a naturally-occurring alanine has been changed to a cysteine, the invention embodies both the peptide containing the naturally-occurring alanine, which has at least partial activity, as well as the peptide containing the substituted

cysteine, which has the preferred activity. Any of the peptides of the invention may be iodinated.

"Homologous" refers to the sequence similarity between two polypeptide molecules or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomeric subunit, e.g., if a position in each of two polypeptide molecules is occupied by leucine, then the molecules are homologous at that position. The homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences. For example, 6 of 10, of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the amino acid sequences Leu-gly-val-ala-gly-pro and Leu-his-tyr-ala-gly-leu share 50% homology.

In addition to substantially full-length polypeptides, the invention also includes fragments of the IGF-I, IGF-II, or IGF-III polypeptides. As used herein, the term "fragment", as applied to a polypeptide, will ordinarily be at least about 5 contiguous amino acids, typically at least about 20 contiguous amino acids, usually at least about 40 contiguous amino acids, and preferably at least about 60 or more contiguous amino acids in length. Fragments of IGF I, II, or III can be generated by methods known to those skilled in the art.

The methods of the invention use IGF-I, IGF-II, IGF-III, functional derivatives of IGF-I, IGF-II, and of IGF-III, combinations thereof, and combinations thereof which also include NGF or functional derivatives of NGF to enhance the survival rate and/or the cholinergic activity of mammalian cells at increased risk of death due to some factor such as disease, injury, or natural aging processes,

or where stimulation of cholinergic activity could have a beneficial effect on the mammal's condition. Some of the functional derivatives utilized by the method of the invention are known; others may be discovered by applying the routine methods disclosed herein. For instance, a functional derivative to be used in any of the various methods of the invention can be any fragment or analog of IGF-I, IGF-II, IGF-III, or any peptide that mimics the biological activity of IGF-I, IGF-II, or IGF-III, as determined by an assay described herein. Examples of such peptides can include IGF fragments containing conservative amino acid insertions, deletions or modified amino acids, cyclic peptides, retro-inverso peptides, or radiolabeled or iodinated peptides, as described herein. The peptides described herein are provided as examples, and are not to be construed as limiting the range of peptides useful for the methods of the invention.

Methods (and compositions) of the invention, e.g., the joint administration of IGF-I and NGF, enhance the survival and neurotransmitter-synthesizing capacity of cholinergic neurons in a previously unknown, complimentary manner.

Survival of a treated neuronal cell denotes maintenance of the cell's viability to an extent greater than that of untreated control cells. Since the preponderance of neuronal cells of the mature CNS are commonly believed to be incapable of cell division, the ability of an agent to promote the survival of such cells may be measured by an assay indicative of cellular trophic response, such as the ornithine decarboxylase assay disclosed herein. Alternatively, one can utilize any other assay which reproducibly indicates relative numbers of surviving cells, such as directly counting cells which stain

as viable cells or which possess other characteristics of viable neurons, or assaying incorporation of appropriate labeled precursors into mRNA or protein. Where the effect of an added growth factor, functional derivatives, or a combination of growth factors and/or functional derivatives on the functioning of cholinergic neurons is of particular interest, an alternative assay that measures that functioning, such as the choline acetyltransferase or acetyl choline esterase assays disclosed herein, may be utilized.

Any of these approaches may be adapted to test the effect of treatment with growth factors, functional derivatives, or combinations of growth factors and/or functional derivatives on particular subsets of neurons known to be vulnerable in specific degenerative diseases, such as spinal cord cholinergic neurons in amyotrophic lateral sclerosis. A preliminary screen for polypeptides which bind to the IGF or NGF receptors may first be employed to indicate likely candidates for the assays described above, e.g., the cell survival or cholinergic activity assay; disclosed herein is an IGF-I-receptor displacement assay designed for such a purpose. Methods for measuring the ability of NGF or its functional derivatives to bind its receptors are known to those skilled in the art. Those polypeptides which appear to promote cell survival or cholinergic activity under one or more of the above assays may be further tested, by appropriate <u>in vivo</u> administration, for their ability to counteract the degenerative effects of aging, injury or disease in the nervous system or other tissue of an animal.

The use of any polypeptide as a therapeutic raises the issue of stability of the polypeptide after administration to the organism, when it is exposed to the action of various peptidases both within and without the

target tissue. Where lack of such stability is expected to be a problem, certain stability-enhancing modifications disclosed herein may be made to the polypeptide. Other modifications designed to facilitate transport of the polypeptide across the blood-brain barrier may be made to the polypeptide, as disclosed herein.

The method of the invention is useful for therapeutically treating a disorder of a human or other mammal characterized by the death of cells, particularly neural cells, including disorders attributable to a disease or aging of, or injury to, such neuronal cells. The neurotrophic peptides, including the IGFs and/or their functional derivatives, and combinations of IGFs and/or their functional derivatives with NGF or its functional derivatives are useful for the treatment of neurodegenerative diseases such as Alzheimer's disease, stroke, epilepsy, amyotrophic lateral sclerosis and Parkinson's disease, as well as general age-related neuronal loss, conditions which have proven particularly intractable to treatment by alternative methods.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiment(s)

The drawings are first described.

Drawings

Fig. 1 is a graph illustrating the effect of IGF-I on the survival of cholinergic neurons in rat spinal cord cultures.

Fig. 2 is a graph showing the effect of IGF-II and IGF-III on the survival of cholinergic neurons in rat spinal cord cultures.

Fig. 3 is a graph illustrating the effect of certain synthetic peptide fragments of IGF-I and IGF-II on the survival of cholinergic neurons in rat spinal cord cultures.

Fig. 4 is a graph depicting the effect on brain ornithine decarboxylase activity of increasing doses of IGF-I injected into the brains of immature rats.

Fig. 5 is a graph showing the effect on brain ornithine decarboxylase activity of injection of IGF-I or synthetic peptide fragments of IGFs into the brains of immature rats.

Fig. 6 is a graph depicting the effect on brain ornithine decarboxylase activity of injection of IGF-I into the brains of mature rats.

Fig. 7 is a graph illustrating the effect of an IGF-II derivative and of IGF-I on survival of cortical cells, as assessed by leucine incorporation.

Fig. 8 is a graph illustrating the effect of an IGF-II derivative and IGF-I on the survival of cortical neurons, as assessed by morphological characteristics.

Fig. 9 is a graph illustrating the additive effect of NGF (at saturating concentration) and IGF-I on ChAT activity in cultured rat septal cells.

Fig. 10 is a graph illustrating the additive effect of NCF and IGF (at saturating concentration) on ChAT activity in cultured rat septal cells.

Fig. 11 is a graph illustrating the effect of timed addition of NGF and IGF-I on ChAT activity in cultured septal cells.

Fig. 12 is a graph illustrating the effect of NGF and IGF-I on the number of AChE positive cells in septal cultures.

Fig. 13 is a graph illustrating that the relationship between cell number and relative fluorescence is linear.

Fig. 14 is a graph illustrating the effect of carboxy-terminal IGF-I and IGF-II peptides, and functional derivatives thereof, on the total number of surviving cells.

Fig. 14a is a graph illustrating the effect of carboxy-terminal IGF-I and IGF-II peptides, functional derivatives thereof, as well as a "scrambled" peptide, on the total number of surviving cells.

Fig. 15 is a graph illustrating that the D-Y modified peptide IGF-II (54-67) is stabilized against degradation when incubated with cells.

Fig. 16 is a graph illustrating the effect of peptide TYCAPAKSE on cortical neuronal cell survival.

Fig. 17 is two photographs illustrating the effect of peptide TYCAPAKSE (SEQ ID NO:1) on neurite outgrowth: a) no peptide; b) 100 μM peptide.

Fig. 18 is a graph illustrating the effect of IGF-I, IGF-II, and IGF-III on choline acetyltransferase activity in spinal cord cells cultured in the absence of serum.

Fig. 19 is a graph illustrating the effect of IGF-I and IGF-II on cortical cell survival.

Fig. 20 is a graph illustrating the effect of IGF-I and IGF-II on cortical cell survival.

Fig. 21 is a graph illustrating the effect of IGF-I, (des 1-3)IGF-I, R3 IGF-I, and IGF-II in promoting motoneuron survival *in ovo*.

<u>The Peptides</u>

The present invention is directed, <u>inter alia</u>, to the modification of neuroactive polypeptides such as IGF-I and IGF-II and their functional derivatives, and their use, both with and without accompanying administration of NGF or

functional derivatives of NGF, as therapeutics for certain neurological diseases or disturbances characterized by increased vulnerability of neurons to dying. A "neuroactive polypeptide" or "growth factor" is defined as a polypeptide which exerts a survival enhancing effect on neuronal cells: e.g., the IGFs, e.g., IGF-I and IGF-II, Nerve Growth Factor (NGF), Epidermal Growth Factor, Fibroblast Growth Factor, and insulin. A "functional derivative" of a polypeptide is a compound which is a fragment, an analog, or an analog of a fragment of that molecule and which possesses the desired biological activity, herein defined as the ability to promote survival and/or cholinergic activity of neuronal cells. A "fragment" of a polypeptide refers to any polypeptide subset of that polypeptide. An "analog" of a polypeptide refers to a molecule having biological activity but possessing some structural differences compared to the polypeptide: e.g., an altered amino acid sequence, or the presence of additional chemical moieties not normally a part of the molecule. Such moieties (introduced, for example, by acylation, alkylation, cationization, or glycosylation reactions) may improve the molecule's solubility, absorption, transport, biological half-life, etc. Alternatively, or in addition, some moieties may decrease the toxicity of the molecule, or eliminate or attenuate any undesirable side effect of the molecule. Moieties capable of mediating such effects are disclosed in <u>Remington's Pharmaceutical Sciences</u> (Mack Pub. Co., Easton, PA, 1980). Although some derivatives of IGF-I, IGF-II, and NGF may be inoperative alone or in combination, a person skilled in the art disclosed herein can recognize which are operative and which are not, as will be explained in more detail below. A "transmitter enhancer" is a polypeptide that causes an increase in the level of a transmitter. NGF is an example

of a transmitter enhancer. A "transmitter" is a neurotransmitter, e.g., acetyl choline. A "transmitter-specific enzyme" is an enzyme present in neurons and involved in transmitter metabolism, e.g., in the case of cholinergic neurons, acetyl choline esterase (AChE) or choline acetyl transferase (ChAT). A "neuronal cell" is a neuron.

Some of the compounds within the scope of this invention are depicted in Table 1, which shows the amino acid sequences (expressed using single-letter abbreviations as defined in Table 2) of IGF-I, IGF-II, and a number of functional derivatives of IGF-I and IGF-II. These derivatives were selected for study on the basis of one or more of the following criteria, which are related to the ability to bind to IGF-I or IGF-II receptors, and thus are useful for

Table 1

IGF PEPTIDE SEQUENCES

Peptide Name	Sequence	Source	Cat. #
Human IGF-I (Somatomedin-C)	GPETL CGAEL VDALQ FVCGD RGFYF NKPTG YGSSS- -RRAPQ TGIVD ECCFR SCDLR RLEMY CAPLK PAKSA	AMGEN[1]	14010
Human IGF-I (Somatomedin-C)	GPETL CGAEL VDALQ FVCGD RGFYF NKPTG YGSSS- -RRAPQ TGIVD ECCFR SCDLR RLEMY CAPLK PAKSA	PENINSULA[2]	9010 Lot 15578
IGF-I(4-70) (Human Brain IGF)	TLCGAEL VDALQ FVCGD RGFYF NKPTG YGSSS- -RRAPQ TGIVD ECCFR SCDLR RLEMY CAPLK PAKSA	KABIGEN[3] KABIGEN[3]	Lot 88:101G Lot S:25
IGF-I(24-41)	YFNKP TGYGS SSRRA PQT	PENINSULA[2]	7308 Lot 007942
	YFNKP TGYGS SSRRA PQT	BACHEM[4]	PGRO 080 Lot F297
	YFNKP TGYGS SSRRA PQT	Synthetic[5]	
IGF-I(30-41)	GYGSS SRRAP QT	PENINSULA[2]	7306 Lot 003251
IFG-I(62-70)	APLKP AKSA	PENINSULA[2]	7318 Lot 015726
IGF-I(24-32)	YFNKP TGYG	Synthetic[5]	
IGF-I(24-41)-AMIDE	YFNKP TGYGS SSRRA PQT-$\underline{NH}_2$	Synthetic[6]	
IGF-I(33-41)-AMIDE	SSSRR APQT-$\underline{NH}_2$	Synthetic[6]	
48-Acm-IGF-I(42-57)-AMIDE	Acm GIVDE CCFRS CDLRR L-$\underline{NH}_2$	Synthetic[7]	

miscaexn

Table 1, continued

Peptide Name	Sequence	Source	Cat. #
IGF-I(33-41)	SSSRR APQT	Synthetic[5]	
IGF-I(28-41)	PTGYG SSSRR APQT	Synthetic[5]	
IGF-I(27-36)	KPTGY GSSSR	Synthetic[5]	
IGF-II(54-67)	ALLET YCATP AKSE	PENINSULA[2]	7308 Lot 010718
IGF-II(62-67)	TPAKS E		
IGF-II(33-40)	SRVSR RSR	PENINSULA[2]	7304 Lot 016905
IGF-II	AYRPS ETLCG GELVD TLQFV CGDRG FYFSR PASRV	COLLABORATIVE[8]	LOT 89-0172
Somatomedin-A	SRRSR GIVEE CCFRS CDLAL LETYC ATPAK SE	COLLABORATIVE[8]	LOT 89-0401

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1 Amgen, Thousand Oaks, CA 91320
2 Peninsula Laboratories, Belmont, CA 94002
3 Kabigen AB, S-112 87, Stockholm, Sweden
4 Bachem, Inc., Torrance, CA 90505
5 Synthesized on a Biosearch Solid Phase Peptide Synthesizer Model 9600 using Fmoc-Amino Acids linked to p-alkoxybenzyl alcohol resins supplied by Bachem Bioscience, Inc. Philadelphia, PA 19104.
6 Synthesized on a Biosearch Solid Phase Peptide Synthesizer Model 9600 using 4-(2', 4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy Resin (AΦ4719) supplied by Novabiochem, AG Laufelfingen, Switzerland.
7 Synthesized on a Biosearch Solid Phase Peptide Synthesizer Model 9600 using the resin identified in footnote [6] Acm=Acetamidomethyl substituent on the cysteine side-chain sulfur atom.
8 Collaborative Research, Inc., Bedford, MA 01730

identifying additional functional derivatives of the invention: (1) conservation of amino acid sequence among species; (2) presence of "conservative" amino acid substitutions among species (i.e., amino acids with similar shape, charge or other salient characteristics); (3) receptor shielding of tyrosine residues from radioiodination (Maly and Luthi, J. Biol. Chem. 263:7068-7072 (1988); (4) predominance of hydrophilic residues, suggesting the location of a receptor-binding domain on the surface of the polypeptide, a presumptive requirement for receptor interaction; and (5) consideration of hydrophobic and polar regions of three-dimensional models (e.g., Blundell et al., Fed. Proc. 42:2592-2597 (1983)) and identifying therefrom regions which are possible binding sites. Analogous factors can be applied in the design of NGF functional derivatives.

Since the ability of peptides to penetrate the blood-brain barrier is related to their lipophilicity or their net ionic charge, suitable modifications of these peptides (e.g., by substituting pentafluorophenylalanine for phenylalanine, or by conjugation to cationized albumin) to increase their transportability (Kastin et al, Pharmac Biochem. Behav. 11:713-716 (1979); Rapoport et al., Science 207:84-86 (1980); Pardridge et al., Biochem. Biophys. Res. Commun. 146:307-313 (1987); Riekkinen et al., Peptides 8:261-265 (1987)) may be important for their bioavailability following administration outside the blood-brain barrier, and these modifications are within the scope of the invention. In addition, since bioavailability of peptides may be limited by their susceptibility to degradation by proteases and peptidases (Littlewood, et al., Neurochem Int. 12:383-389 (1988)),modifications of these peptides (e.g., replacement of L-amino acids with D-amino acids) to increase their

metabolic stability (Coy et al., 1976) may also be important for their therapeutic efficacy, and these modified peptides are also within the scope of the invention.

Functional derivatives of the invention include, among others, peptides that vary from the native IGF or NGF molecules in any one or more of the following ways:

1. Chemical modification of the amino and carboxyl groups present at the respective ends of the peptides.

2. Replacement of one or more of the amino acid residues in the native sequence with biologically compatible other amino acid residues.

3. Replacement of one or more of the amino acid residues in the native sequence with chemically modified, biologically compatible other amino acid residues.

4. Deletion of one or more of the amino acid residues in the native sequence.

5. Repetition of one or preferably a sequence of several amino acid residues in the native sequence, with or without chemical modification to, or replacement or deletion of, one or more of the members of the sequence.

6. Cyclization, that is, joining the amino and carboxyl ends of the linear peptide.

7. Linkage of a IGF-I, IGF-II, IGF-III, CTNF, NGF, or functional derivatives of any of IGF-I, IGF-II, IGF-III, CTNF, or NGF with another molecule such as a polypeptide (e.g., another fragment of IGF-I, IGF-II, IGF-III, CTNF, or NGF) or a carbohydrate, by means of a disulfide, peptide, ester or other covalent bond.

8. Depsi peptide analogs.

9. Retro-Inverso peptides.

Examples of some of the functional derivatives of the invention are shown in Table 3, and further described in

Table 4. The amino acid and molecular mass analyses of the Table 3 peptides are summarized in Table 4.

The invention also utilizes as a preferred subgroup within the IGF functional derivatives described above, those functional derivatives having the sequence: R_1- AA_1- AA_2- AA_3- AA_4...AA_n-R_2, wherein AA_1, AA_2, AA_3, AA_4...AA_n are amino acid residues of IGF or of the IGF-peptide subsets or are conservative replacements for them as defined in Table 2, and n is any integer from 5 to 70 for IGF-I functional derivatives and 5-67 for IGF-II functional derivatives. R_1 is attached to the amino group of AA_1 and selected from the group of hydrogen, lower (C_{1-6}) alkyl, lower alkyl carbonyl, lower alkenyl, lower alkynyl, formyl, lower (C6-10) aryl, aroyl, aryloxy-carbonyl, aralkyloxy-carbonyl, lower alkyloxycarbonyl, benzoyl, 1- or 2-thenoyl, nicotinoyl, dihydronicotinoyl, N-alkyldihydronicotinoyl, isonicotinoyl, and N-alkyldihydroisonicotinoyl. The carboxyl-terminal substituent (R_2) of the peptides is selected from the following: OH; NH_2; OR_3, wherein R_3 is a lower alkyl or a lower aryl; OR_3OH, wherein R_3 is defined as above; and NH-R_3 or $N(CH_3)R_3$, wherein R_3 is defined as above. Alternatively, the carboxyl group of the carboxyl-terminal amino acid may be replaced by any one of $-PO_3H_2$, $B(OH)_2$, $-CH_2OH$, $-SO_3H$ or a 5-tetrazole group.

Table 2

CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid	Code	Replace with
Alanine	A	D-Ala, Gly, Aib, β-Ala, Acp, L-Cys, D-Cys, or delete
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn or delete
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln, or delete
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln, or delete
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr, or delete
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp or delete
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln, or delete
Glycine	G	Ala, D-Ala, Pro, D-Pro, Aib, β-Ala, Acp or delete
Isoleucine	I	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met, or delete
Leucine	L	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met or delete
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn or delete
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val or delete

Table 2 (continued)

CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid	Code	Replace with
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa or delete
Proline	P	D-Pro, L-I-thiazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid (Kauer, U.S. Patent (4,511,390) or delete
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys, or delete
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val or delete.
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His or delete
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG or delete

The invention also utilizes as a preferred subgroup within the NGF functional derivatives described above, those functional derivatives having the sequence: R_1-AA_1-AA_2-AA_3-AA_4...AA_n-R_2, wherein AA_1, AA_2, AA_3, AA_4...AA_n are amino acid residues of NGF or its functional derivatives or are conservative replacements for them as defined in Table 2, and n is an integer corresponding to the number of amino acid residues in NGF or in a functional derivative thereof. R_1 is attached to the amino group of AA_1 and selected from the group of hydrogen, lower (C_{1-6}) alkyl, lower alkyl carbonyl, lower alkenyl, lower alkynyl, formyl, lower (C_{6-10}) aryl, aroyl, aryloxy-carbonyl, aralkyloxy-carbonyl, lower alkyloxycarbonyl, benzoyl, 1- or 2-thenoyl, nicotinoyl, dihydronicotinoyl, N-alkyldihydronicotinoyl, isonicotinoyl, and N-alkyldihydroisonicotinoyl. The carboxyl-terminal substituent (R_2) of the peptides is selected from the following: OH; NH_2; OR_3, wherein R_3 is a lower alkyl or a lower aryl; OR_3OH, wherein R_3 is defined as above; and NH-R_3 or $N(CH_3)R_3$, wherein R_3 is defined as above. Alternatively, the carboxyl group of the carboxyl-terminal amino acid may be replaced by any one of $-PO_3H_2$, $-B(OH)_2$, $-CH_2OH$, $-SO_3H$ or a 5-tetrazole group.

The amino-terminal amino group and/or the lysine, serine or threonine side chains occurring within the peptide may optionally be acylated by formyl, acetyl, propionyl, and similar lower alkylacyl residues or by aryl or heterocyclic acyl residues such as benzoyl, thenoyl, nicotinoyl, isonicotinoyl, N-alkylnicotinoyl and their dihydro and tetrahydro derivatives. Such modifications would be expected to enhance the blood-brain barrier permeability of the therapeutic agent (Creveling et al., Experientia 25:26-27 (1969); Bodor et al., Science 214:1370-1372 (1981)).

In peptide sequences containing proline, glutamic acid, or aspartic acid at the amino-terminus, the amino terminal amino acid may optionally be replaced by L-pyroglutamic acid.

The fragment polypeptides of IGF-I, IGF-II, and NGF are subsets of the IGF-I, IGF-II, and NGF molecules (respectively) containing fewer amino acid residues than the native molecules. Preferred IGF sequences are of 5-40 residues and most preferred are sequences of 6-25 residues. A portion of the amino acids of the fragments may be substituted with conservative replacements, deletions, or insertions that improve the chemical or biological stability of the product peptides or improve their transport across the blood-brain barrier. Preferably, no more than 30% and more preferably no more than 20%, of the amino acid residues are replaced or deleted. A listing of suitable conservative replacements is given in Table 2, along with a key to the single-letter abbreviations for the common, naturally-occurring amino acid residues found in proteins. Certain other abbreviations used in Table 2 are herein defined: by Nle is meant norleucine, by Aib is meant aminoisobutyric acid, by AdaA is meant β-adamantylalanine, by AdaG is meant α-adamantylglycine, by homo-Arg is meant L-homoarginine, by D-homo-Arg is meant D-homoarginine, by Acp is meant ε-aminocaproic acid, by Chg is meant L-α-cyclohexylglycine, by D-Y is meant D-Tyrosine, and by allo-Thr is meant L-allothreonine. Additionally, by Cha is meant β-cyclohexyl-alanine, by Me is meant methyl (CH_3), by Orn is meant ornithine, by pyro-Glu is meant the pyroglutamyl group, by Met(O) and D-Met(O) are meant the sulfoxides derived from L- and D-methionine, respectively, by β-Ala is meant β-alanine, by Acm is meant acetamidomethyl, by L-Dopa is meant 3-(3,4-

dihydroxyphenyl)-L-alanine, and by Bpa is meant 4-benzoylphenylalanine.

The symbolism and abbreviations used are otherwise those recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature, "Nomenclature and Symbolism for Amino Acids and Peptides, Recommendations 1983" J. Biol. Chem. 260:14-42 (1985). As is conventional, these same symbols are used to define the corresponding residues of the amino acids when they are linked into a peptide chain. Where the amino acid residue has isomeric forms, it is the L-form of the amino acid that is represented unless otherwise expressly indicated. In accordance with conventional representation, the amino group at the N-terminus of each peptide appears to the left and the carboxyl group at the C-terminus to the right.

Besides the amino acid substitutions suggested above, other methods of improving transport of the polypeptide across the blood-brain barrier, such as chemical modification of the polypeptide, may be employed. In any chemical modification procedure, the polypeptide may first be attached to its receptor in order to protect and maintain the receptor-binding site structure during the chemical modification process, which can comprise, for example, cationization (according to the method, for example, of Pardridge et al., 1987) or glycosylation (according to the method of Schwartz et al., Arch. Biochem. Biophys. 181:542-549 (1977)).

Cyclic Peptides

The invention also utilizes as a preferred subgroup within the IGF functional derivatives described above, cyclic peptides, preferably of 5-40 amino acid residues, and most preferably of 6-25 amino acid residues. Such peptides are preferably modeled after the looped domains of the IGF

molecules. Such loops may be a consequence of natural disulfide bond formation, while others are a consequence of the folding of the protein as it achieves a minimal energy conformation or a receptor-induced conformation to permit binding. As stated above, cyclization can be effected by joining the amino and carboxyl ends of a linear peptide, either directly to form an amide (lactam) bond (Example 25-B), or by disulfide bond formation employing terminal cysteine groups. Any internal cysteine groups present are preferably selectively blocked before cyclization and may be unblocked afterward using well-established procedures (Example 25A). Alternatively, internal cysteines may be replaced by an amino acid which would be expected to have a minimal influence on peptide conformation, e.g. alanine, which is frequently used in scanning mutagenesis studies.

Examples of preferred cyclic peptides include those derived by cyclization of the following monomeric peptides via disulfide bond formation of the terminal cysteine groups:

CALLETYCATPAKSEC	(SEQ ID NO:6)
CTYCATPAKSEC	(SEQ ID NO:7)
CEPYCAPPAKSEC	(SEQ ID NO:8)
CTYCAPAKSEC	(SEQ ID NO:9)
CALLETDYCATPAKSEC	(SEQ ID NO:47)
CTDYCATPAKSEC	(SEQ ID NO:48)
CTDYCAPAKSEC	(SEQ ID NO:49)
CTYTAPAKSEC	(SEQ ID NO:10)
CALLETYATPAKSEC	(SEQ ID NO:11)
CRRLEMYCAPLKPAKSAC	(SEQ ID NO:12)
CGYGSSSRRAPQTC	(SEQ ID NO:13)
CYFNKPTGYGC	(SEQ ID NO:14)
CYFNKPTGYGSSSRRAPQTC	(SEQ ID NO:15)
CKPTGYGSSSRC	(SEQ ID NO:16)

An example of a cyclic peptide formed by amide bond formation is the following:

Cyclic (TYCAPAKSE) (SEQ ID NO:1)

Examples of preferred cyclic peptides based on looped domains of the IGF-I and IGF-II molecules are the following:

IGF I

GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTG
IVDECCFRSCDLRRLEMYCAPLKPAKSA (SEQ ID NO:17)

LOOP PEPTIDES PROPOSED:

1. Using Cys present in IGF I.

a)	CGCELVDALQFVC	6-18[2]	(SEQ ID NO:18)
b)	CDLRRLEMYC	52-61	(SEQ ID NO:19)
c)	CCFRSCDLRRLEMYC	47-61	(SEQ ID NO:20)
d)	CDLRRLEMYCCPLKPAKSE	52-70	(SEQ ID NO:21)
e)	CCFRSC	47-52	(SEQ ID NO:22)
f)	CFRSC	48-52	(SEQ ID NO:23)
g)	CGCELVDALQFVC	6-18	(SEQ ID NO:18)
	CCFRSCDLRRLEMYC	47-61	(SEQ ID NO:20)

2. Using extra Cys.

h)	CGPETLC	C+1-6	(SEQ ID NO:26)
i)	CGYGSSSRRCPQTGIVDEC	C+30-47	(SEQ ID NO:27)

[2] Numbers refer to position of amino acids in corresponding naturally occurring IGF-I.

j) CGDRGFYFNKPTC 21-31+C (SEQ ID NO:28)

k) CCPLKPAKSAC 61-70+C (SEQ ID NO:29)

l) CDLRRLEMY*APLKPAKSAC[3] 52-70+C (SEQ ID NO:30)

IGF II

AYRPSETLCGGELVDTLQFVCGDRGFYFSRPASRVSRRSR GIVEECCFRSCDLALLETYCATPAKSE (SEQ ID NO:31)

LOOP PEPTIDES PROPOSED[4]:

1. Using Cys present in IGF II.

a) CGGELVDTLQFVC 9-21[5] (SEQ ID NO:32)

b) CDLCLLETYC 51-60 (SEQ ID NO:33)

c) CCFRSCDLALLETYC 46-60 (SEQ ID NO:34)

d) CDLCLLETYCATPAKSE 51-67 (SEQ ID NO:35)

e) CCFRSC 46-51 (SEQ ID NO:22)

f) CFRSC 47-51 (SEQ ID NO:23)

g) CGGELVDTLQFVC 9-21 (SEQ ID NO:32)

---

[3] * denotes deletion of an amino acid from the corresponding position of naturally occurring IGF-I or IGF-II.

[4] Some of the following peptides contain an Ala--->Cys substitution.

[5] Numbers refer to position of amino acids in corresponding naturally occurring IGF-II.

CCFRSCDLCLLETYC	46-60	(SEQ ID NO:39)

2. Using extra Cys.

h)	CCYRPSETLC	C+1-9	(SEQ ID NO:40)
i)	CRPCSRVSRRSRGIVEEC	C+30-46	(SEQ ID NO:41)
j)	CGDRGFYFSRPC	21-31+C	(SEQ ID NO:42)
k)	CCTPAKSEC	60-67+C	(SEQ ID NO:43)
l)	CDLCLLET*ATPAKSEC	51-67+C	(SEQ ID NO:44)

Retro-inverso Peptides

A retro-isomer of a peptide is defined by a reversal of the direction of the peptide bond while maintaining the side-chain topochemistry. In retro-inverso peptides, D-amino acids are substituted for L-amino acids to retain the overall conformation for biological response and receptor binding similar to the native peptides (Hayward et al., Peptides 1974: Proc. 13th Eur. Peptide Symp., ed. Y. Wolman, pp. 287-297; Goodman et al., Acc. Chem.Res. 12:1-7 (1979)). It has been shown that the retro-inverso peptides introduced well defined conformational constraints and showed limited biodegradation by endopeptidases.

```
 -HN-CH-CO-NH-CH-CO-NH-CH-CO-NH-        Native peptide
     |        |        |
-HN-OC-CH-NH-OC-CH-NH-OC-CH-NH-         Retro D-peptide
        |        |        |
 -HN-CH-NH-OC-CH-NH-OC-CH-CO-NH-        Retro D-peptide
     |        |        |                with modified
                                        end group
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The reversal of the amino- and carboxyl termini in the retro D-peptides reduces the activity in cases where the

terminal group was involved in activity. Modifications may be made at the carboxy- terminus by introducing a 2-alkylmalonate derivative and a 2-alkyl substituted geminal diamine at the amino- terminus. These groups may also be used as bridging residues when a partial or single amide modified retro-inverso segment is incorporated in a native sequence. Partial and selected single amide modified retro peptides may be used to modify the biological activity. Examples of different retro-inverso peptides are depicted here in a general sequence.

gAA -- AA -- AA -- AA -- AA -- mAA	End to end modification
gAA -- AA -- AA --mAA -- AA -- AA	Partial modification
gAA --mAA -- AA -- AA -- AA -- AA	Single amide modification

gAA = 2-substituted geminal diamine amino acid surrogate
mAA = 2-alkyl malonate amino acid surrogate
AA = L-, D- or unusual amino acid based on the design

Retro-inverso peptides are synthesized both by the solution phase segment condensation method and the solid phase method. A general procedure for preparing a geminal diamino and malonyl derivative of alanine is given below.

Synthesis of gAla:
Z-HN-CH(CH_3)-CONH.NH_2 ---> Z-HN-CH(CH_3)-CO-N_3 --->
Z-HN-CH(CH_3)-N=C=O ----> Z-HN-CH(CH_3)-NH.Boc --->
Z-HN-CH(CH_3)-NH_2

Synthesis of mAla:
C_2H_5OOC-CH_2-$COOC_2H_5$ ---> C_2H_5OOC-CH(CH_3)-$COOC_2H_5$--->
HOOC-CH(CH_3)-$COOC_2H_5$

Proposed sequences: The retro-inverso peptides of the following fragments of IGF-I and IGF-II can be made

following generally known peptide procedures. Numbers denote the corresponding amino acid positions of full-length IGF-I (SEQ ID NO:17), or of full-length IGF-II (SEQ ID NO:31), respectively.

<u>IGF-I</u>:

GPETL CGAEL VDALQ FVCGD RGFYF	1-25
AEL VDALQ FVCGD RGFYF	8-25
GPETL CGAEL VDALQ	1-15
GPETL CGAEL	1-10
VDALQ FVCGD RGFYF	11-25
FVCGD RGFYF	16-25
RGFYF NKPTG YGSSS RRAPQ TGIVD	21-45
RRAPQ TGIVD	36-45
YGSSS RRAPQ TGIVD	31-45
NKPTG YGSSS RRAPQ TGIVD	26-45
NKPTG YGSSS RRAPQ	26-40
RGFYF NKPTG YGSSS	21-35
SCDLR RLEMY CAPLK PAKSA	51-70
RLEMY CAPLK PAKSA	56-70
CAPLK PAKSA	61-70
SCDLR RLEMY CAPLK	51-65
SCDLR RLEMY	51-60
RLEMY CAPLK	56-65

<u>IGF-II</u>:

VCGDR GFYFS RPSSR INRRS RGIV	20-44
GFYFS RPSSR INRRS RGIV	26-44
RPSSR INRRS RGIV	31-44
GFYFS RPSSR INRRS	26-40
VCGDR GFYFS RPSSR	20-35
CFRSC DLALL ETYCA TPAKS E	47-67
LALL ETYCA TPAKS E	53-67
TYCA TPAKS E	58-67
CFRSC DLALL ETYCA	47-61
DLALL ETYCA	52-61

TABLE 3

Sequence	Resin used	Purification method* (RT)	Wt. of pure peptide(mg)	SEQ ID NO
TYCAT PAK	Fmoc-Lys(Boc)-resin (1.0g, 0.63meq/g)	I (13.8 min)	35.9	51
LETYC ATP	Fmoc-Pro-resin (0.5g, 0.36meq/g)	I (20.7 min)	6.1	52
CATPA KSE	p-alkoxybenzylalcohol (1.0g, 0.97meq/g)	II (22.8 min)	11.6	53
TdYCAP AKSE	Fmoc-CAPAKSE-resin (0.2g, 0.97meq/g)	III (13.3 min)	9.7	50
YCAPA KSE	Fmoc-CAPAKSE-resin (0.2g, 0.97meq/g)	IV (13.4 min)	14.3	54
YCAPA	p-alkoxybenzylalcohol (1.0g, 0.97meq/g)	V (9.7 min)	16.0	55
TYCAP A	Fmoc-YCAPA-resin (0.3g, 0.97meq/g)	VI (16.6 min)	25.0	56
CAPAK SE	p-alkoxybenzylalcohol (0.4g, 0.97meq/g)	IV (9.1 min)	16.2	24
$TY(I_2)CAP$ AKSE	Fmoc-APAKSE-resin (0.31g, 0.97meq/g)	VII (13.4 min)	17.9	25
EALLE TYCAT PAKSE	Fmoc-Glu(t-Bu)-resin (0.5g, 0.36meq/g)	VIII (12.7 min)	10.8	36
ALLEK YCAKP AKSE	Fmoc-Glu(t-Bu)-resin (0.5g, 0.36meq/g)	IX (14.3 min)	35.0	37
APSTC EYKA	p-alkoxybenzylalcohol (0.5g, 0.97meq/g)	III	9.9	38

* Purification methods by HPLC:
RT = Retention time
Solvent A = water with 0.1% TFA** and B = acetonitrile with 0.1% TFA
Flow rate = 9.5mL/min.(Waters) and 3.5 mL/min.(Vydac)

I. 0-40% of B in 40 min. Column: Waters C8
II. 0-10% of B in 40 min. Column: Waters C8
III. 5-15% of B in 15 min. Column: Vydac C8
IV. 0-10% of B in 10 min. Column: Vydac C8
V. 5-60% of B in 40 min. Column: Vydac C18
VI. 5-60% of B in 60 min. Column: Waters C18
VII. 5-40% of B in 25 min. Column: Vydac C18
VIII. 10-25% of B in 40 min. Column: Waters C8
IX. 10-30% of B in 40 min. Column: Vydac C8

** TFA = trifluoroacetic acid
(I) = iodination

TABLE 4

Sequence	Amino acid analysis Theory (Found)*	Molecular mass Calculated (Found)	SEQ ID NO:
TYCATPAK	Thr 2 (1.96); Ala 2 (2.28) Pro 1 (0.98); Tyr 1 (1.00) Lys 1 (1.04); Cys 1	854.14 (854)	51
LETYCATP	Glx 1 (1.02); Thr 2 (1.74) Ala 1 (1.23); Pro 1 (1.10) Tyr 1 (1.00); Leu 1 (1.14) Cys 1	897.16 (898)	52
CATPAKSE	Glx 1 (1.05); Ser 1 (0.99) Thr 1 (1.15); Ala 2 (2.09) Pro 1 (0.99); Lys 1 (0.87) Cys 1	805.00 (806)	53
TdYCAPAKSE	Glu 1 (0.86); Ser 1 (0.90) Thr 1 (1.30); Ala 2 (2.04) Pro 1 (0.86); Tyr 1 (1.00) Lys 1 (1.07)	969.00 (970)	50
YCAPAKSE	Glu 1 (0.94); Ser 1 (0.86) Ala 2 (1.96); Pro 1 (0.93) Tyr 1 (0.93); Lys 1 (1.30) Cys 1	867.99 (868)	54
YCAPA	Ala 2 (2.09); Pro 1 (0.96) Tyr 1 (0.98); Cys 1	523.00 (524)	55
TYCAPA	Thr 1 (1.18); Ala 2 (2.00) Pro 1 (0.95); Tyr 1 (0.96) Cys	624.00 (625)	56
CAPAKSE	Glu 1 (0.92); Ser 1 (0.88) Ala 2 (2.22); Pro 1 (1.08) Lys 1 (1.09); Cys 1	704.00 (705)	24
TY(I_2)CAPAKSE	Glx 1 (0.75); Ser 1 (0.99) Thr 1 (1.02); Ala 2 (2.00) Pro 1 (1.02); Tyr 1 (0.99) Lys 1 (1.28); Cys 1	1220.00 (1221)	25
EALLETYCATPAKSE	Glx 3 (3.04); Ser 1 (0.91) Thr 2 (1.84); Ala 3 (3.03) Pro 1 (0.92); Tyr 1 (0.98) Leu 2 (2.18); Lys 1 (1.19) Cys 1	1625.00 (1626)	36
ALLEKYCAKPAKSE	Glx 2 (2.00); Ser 1 (0.81) Ala 3 (2.96); Pro 1 (0.99) Tyr 1 (0.95); Leu 2 (2.00) Lys 3 (3.07); Cys 1	1551.06 (1552)	37

Table 4, continued

Sequence	Amino acid analysis Theory (Found)*	Molecular mass Calculated (Found)	SEQ ID NO:
APSTCEYKA	Glx 1 (1.02); Ser 1 (0.97) Thr 1 (0.89); Ala 2 (2.21) Pro 1 (0.89); Tyr 1 (0.94) Lys 1 (1.14); Cys 1	969.00 (969)	38

* Cysteine was not determined

Uses of the Peptides

As described more fully below, the present invention provides novel uses of IGF-I and IGF-II and their functional derivatives, and of IGF-I, IGF-II, and their functional derivatives in combination with NGF and its functional derivatives, as agents for the treatment of diseases or disturbances characterized by an increased risk of cell death, including in particular, neuronal cell death. The bioactivity of each polypeptide (or combination of polypeptides) of the invention may be conveniently assayed by a brain ornithine decarboxylase assay, a spinal cord choline acetyl transferase assay, a cultured septal cell assay, or a cultured cortical cell assay, all of which are described in detail below. Alternatively, the polypeptides may first be screened by a receptor-growth factor displacement assay, e.g., the receptor-IGF-I displacement assay described below, which measures the polypeptide's ability to displace labelled IGF-I bound to receptors in homogenized brain tissue. This assay has been demonstrated to correlate with the polypeptide's bioactivity as measured by the two enzymatic assays. As described in the examples below, these assays disclose previously unknown bioactivity of IGF-I, IGF-II, IGF-III and some functional derivatives of these molecules both alone, and in combination with NGF or functional derivatives of NGF. Thus, the peptides of this invention should be useful for administration to humans or other mammals who suffer from neurological diseases or disturbances characterized by increased risk of neuronal cell death, as described above. These neurological diseases or disturbances include but are not limited to: Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, stroke, and concussive or penetrating injuries of the brain or spinal cord.

The formulations of this invention are useful for parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol, scarification, and also for oral, buccal, rectal or vaginal administration. The compositions can be formulated for parenteral administration to humans or other mammals in therapeutically effective amounts (e.g., amounts which eliminate or reduce the patient's pathological condition) to provide therapy for the neurological diseases described above.

The compounds provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable nontoxic excipients and carriers. As noted above, such compositions may be prepared for use as parenteral administration, particularly in the form of liquid solutions or suspensions; for oral administration, particularly in the form of tablets or capsules; or intranasally, particularly in the form of powders, nasal drops, or aerosols.

The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical art, for example, as described in <u>Remington's Pharmaceutical Sciences</u>. Formulations for parenteral administration may contain as common excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be useful excipients to control the release of the peptides. Other potentially

useful parenteral delivery systems for these peptides include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The materials of this invention can be employed as the sole active agent in a pharmaceutical or can be used in combination with other active ingredients, e.g., other growth factors which could facilitate neuronal survival in neurological diseases, or peptidase or protease inhibitors.

The concentration of the compounds described herein in a therapeutic composition will vary depending upon a number of factors, including the dosage of the drug to be administered, the chemical characteristics (e.g., hydrophobicity) of the compounds employed, and the route of administration. In general terms, the compounds of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. Typical dose ranges are from about 1 μg/kg to about 1 g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage of drug to be administered is likely to depend on such variables as the type and extent of progression of the neurological disease, the overall health status of the particular patient, the relative biological efficacy of the compound selected, the

formulation of the compound excipients, and its route of administration.

The present invention will be further illustrated by the following examples. These examples are not be construed as limiting the scope of the invention, which is to be determined solely by the appended claims.

EXAMPLE 1

Recombinant human IGF-I, IGF-II, and IGF-III, as well as several chemically synthesized peptides consisting of partial sequences of IGF-I or IGF-II, were obtained from commercial sources as indicated in Table 1. ^{125}I-labeled [Threonine^{59}]IGF-I was obtained from Amersham (Arlington Heights, IL). Other peptides consisting of partial sequences of IGF-I or IGF-II were chemically synthesized using Fmoc chemistry on a Milligen Biosearch Model 9600 Peptide Synthesizer, and purified on Hewlett-Packard Models 1050 and 1090M HPLCs according to the method of Hudson, J. Org. Chem. 53:617-624 (1988). Fmoc amino acids, BOP (Castro's reagent), and resins were purchased from Biosearch (San Raphael, CA 94901) and Bachem Bioscience, Inc. (Philadelphia, PA 19104). Solvents were purchased from Burdick and Jackson (Muskegon, MI 49442). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO 63178).

Brain tissue containing the cerebral cortex and cerebellum was dissected from adult Sprague-Dawley rats (Hilltop Lab Animals, Inc. Scottsdale, PA) and homogenized at low power for 5 minutes in a Brinkmann Polytron homogenizer (Westbury, NY) containing 50 volumes of ice-cold buffer consisting of 10 mM HEPES, 0.5% BSA, 0.0125% NEM, 0.025% bacitracin, and 100 KIU/ml aprotinin, pH 7.6 (Bohannon et al., Endocrinology 119:943-945 (1986). Following homogenization, the tissue was collected after

centrifugation at 7800 x g for 20 minutes and resuspended in 10 volumes of assay buffer. Tissue (50 μl), 100 μl ^{125}I-[Threonine^{59}]IGF-I (20 pM), and 50 1 of buffer or peptides of varying concentration were added to 96-well plates and incubated on ice for 3 hours. After the incubation period, the tissue was collected on Whatman GF/C filters that had been pre-soaked in 0.01% polyethylenimine and washed four times with ice-cold assay buffer using a Brandel cell harvester (Gaithersburg, MD). The filters were removed and the bound ^{125}I-[Threonine^{59}]IGF-I was measured using a Beckman Model 5500B Gamma Counter.

Table 5 summarizes the results of the ^{125}I-[Threonine^{59}]IGF-I displacement assay utilizing native IGFs and IGF fragments. The results demonstrate that, while IGF-I and IGF-III are potent displacers of ^{125}I-[Threonine^{59}]IGF-I, IGF-II is essentially inactive, indicating that the assay is selective for the identification of IGF-I-like molecules. In this assay, IGF-I(24-41) alone or in combination with IGF-II(54-67) were active in displacing ^{125}I-[Threonine^{59}]IGF-I. IGF-II(54-67) alone, and several other fragments listed in Table 5 were not significantly effective displacers of ^{125}I-[Threonine^{59}]IGF-I.

EXAMPLE 2

Brains were removed intact from adult Sprague-Dawley rats, frozen on powdered dry ice, and cut into 20μm sections (at the level of the cerebellum and brain stem) which were

Table 5

IGF-I RECEPTOR COMPETITION ASSAY SUMMARY

PEPTIDE (CONC.)	PERCENT MAX.	BOUND (SD)
IGF-I (10 pM)	100	(1.1)
IGF-I (40 nM)	9.6	(0.7)
IGF-II (40 nM)	92.1	(0.7)
IGF-III (40 nM)	17.6	(2.6)
IGF-I(24-41) (100 μM)	44	(7)
IGF-I(24-41) (50 μM)	99	(6)
IGF-I(24-41) (50 μM) +		
IGF-II(54-67)(50 μM)	49	(11)
IGF-II(54-67)(100μM)	94	(6)
IGF-I(62-70) (100 μM)	83	(20)
IGF-I(30-41) (100 μM)	94	(1.4)
IGF-II(62-67) (100 μM)	83	(21)
IGF-II(33-40) (1mM)	92	(1.8)

thaw-mounted onto gelatin-coated glass microscope slides (Herkenham and Pert, J. Neurosci. 2:1129-1149 (1982)). Using a modification of the method of Bohannon et al. (1986), the tissue sections were covered with 250 μl of HEPES assay buffer (see Example 1) containing 0.01 nM ^{125}I-[Threonine^{59}]IGF-I alone or in combination with unlabeled IGF-I, IGF-II, or synthetic peptide fragments thereof. The sections were incubated at 4°C for 24 hours and then rinsed in three 1-minute changes (200 ml each) of ice-cold HEPES assay buffer. The tissue sections were then wiped off the slides with filter paper, and the tissue-bound radioactivity was measured in a Beckman Model 5500B Gamma Counter.

In this assay, in contrast to the assay described in Example 1, ^{125}I-[Threonine^{59}]IGF-I binding was potently displaced by both IGF-I and IGF-II, indicating the utility of this assay for detecting potentially active derivatives of both of these molecules (Table 6). ^{125}I-[Threonine^{59}]IGF-I binding was displaced by IGF-II(33-40), but not by IGF-II(54-67).

EXAMPLE 3

The activity of IGF-I, IGF-II, or synthetic peptide derivatives of these molecules was assayed on dissociated cultures of 14-day embryonic rat spinal cord neurons. The spinal cord neurons were obtained from trypsin-dissociated spinal cords, plated, incubated with peptides, and subsequently (48 hr later) assayed for choline acetyltransferase activity as described by McManaman et al., Dev. Biol. 125:311-320 (1988).

In this assay, IGF-I was found to produce a substantial, dose-dependent increase in choline acetyltransferase activity (Fig. 1), suggesting that IGF-I

Table 6

PEPTIDE	PERCENT MAX. BOUND
IGF-I (4 pM)	91
IGF-I (400 pM)	30
IGF-II (200 nM)	50
IGF-II (400 nM)	23
IGF-II (33-40)(1 mM)	76
IGF-II (33-40)(.10 mM)	82
IGF-II (54-67)(.25 mM)	167
IGF-II (54-67) (.025 mM)	132

can dramatically enhance the cholinergic activity and/or survival of spinal cord cholinergic neurons. Furthermore, IGF-II and IGF-III were found to be active in the spinal cord assay (Fig. 2). In addition, IGF-I(24-41) and IGF-II(33-40) were also found to produce a dose-dependent increase in choline acetyltransferase activity, indicating that each peptide is an active IGF functional derivative (Fig. 3).

EXAMPLE 4

The in vivo activity of IGF-I, IGF-II or synthetic peptide derivatives of these molecules was tested using a biochemical marker for CNS neurotrophic activity, the induction of brain ornithine decarboxylase. The induction (i.e. increased activity) of ornithine decarboxylase has been reported to be a general marker for the actions of a variety of trophic factors. (Schwartz et al., Dev. Brain Res. 1:403-413 (1981); Kanje et al., Brain Res. 381:24-28 (1986); Russell et al., Life Sci. 19:1297-1306 (1976);MacDonnell et al. Proc. Natl. Acad. Sci. USA 74, 4681-4684 (1977); Rinehart et al. Proc. Natl. Acad. Sci. USA 82, 4365-4368 (1985)).

Sprague-Dawley rats, 4 days old, were injected intracerebrally (in the area of the lateral ventricle) with 5 µl of 0.1 M phosphate-buffered saline (PBS) containing IGF-I, IGF-II or a synthetic peptide derivative (1.25-2.5 µg dose, with 6 animals per treatment group). After 6 hours, the brains were removed, and ornithine decarboxylase was assayed essentially as described by Lewis et al., Proc. Natl. Acad. Sci. USA 75:1021-1023 (1978).

Administration of IGF-I produced a dose-dependent increase in brain ornithine decarboxylase activity (Fig. 4). In addition, both IGF-I(24-41) and IGF-II(54-67) increased brain ornithine decarboxylase activity (Fig. 5;

these peptides are referred to in Fig. 5 as IGF-I (2-4) and IGF-I(5-6), respectively).

EXAMPLE 5

To determine whether the induction of brain ornithine decarboxylase by IGF-I was limited to developing animals, IGF-I was also injected intraventricularly into the lateral ventricles of adult Sprague-Dawley rats. After 6 hours, the brains were removed, dissected into several regions (cerebral cortex, medial septum, and hippocampus), and then assayed for ornithine decarboxylase activity as described in Example 4. As shown in Fig. 6, IGF-I stimulated ornithine decarboxylase activity in all brain regions assayed. This result indicates that IGF-related molecules have potential utility in widespread regions of the brain.

EXAMPLE 6

The ability of IGF-I and a synthetic derivative of IGF-II (IGF-II(54-67)) to increase the incorporation of $[^3H]$-leucine and to promote the survival of neurite bearing cells was examined in cultured rat cortical cells (the numbers "54-67" in IGF-II indicate the fragment includes amino acid residues 54-67 of native IGF-II). IGF-II(54-67), like IGF-I, increased $[^3H]$-leucine incorporation in low density 24 hour mixed cortical cultures, as shown in Fig. 7. IGF-II(54-67) also displayed IGF-I-like survival-promoting activity in that it increased the survival of cortical neurons (as determined by the presence of neurite bearing cells), as shown in Fig. 8.

Measurements were performed, using standard techniques known to those skilled in the art, on dissociated cortical cells obtained from day 18-19 embryonic rats. Cells were seeded at $1.5 \times 10^4/cm^2$ on poly-l-ornithine-laminin coated plastic tissue culture cells in serum-free N2

medium (Bottenstein et al. Proc. Natl. Acad. Sci. U.S.A. 76: 514-517 (1978)). [^{3}H]-leucine was added to cells at plating for the incorporation assay. Cultures were terminated 24 hours after plating and measured for either [^{3}H]-leucine incorporation or for the number of neuritic cells by microscopic examination.

EXAMPLE 7

The effect of simultaneous administration of IGF-I and NGF on ChAT activity was assayed in cultured septal neurons. ChAT is the initial enzyme in the synthesis of the neurotransmitter, acetylcholine, and is a specific biochemical marker for cholinergic neurons. Assay of this enzyme may be used as an indication of the effects of IGF (and other factors) on the survival of cholinergic neurons and/or regulation of this enzyme. An additive increase in ChAT activity was seen with saturating concentrations of NGF combined with saturating or sub-maximal concentrations of IGF-I, as shown in Fig. 9. In Fig. 9 open squares represent IGF-I, diamonds indicate IGF-I + 2nM NGF, open circles indicate 2nM NGF, and the horizontal line at 403 DPM represents uninduced cells. A similar additive effect was seen when saturating concentrations of IGF-I were combined with saturating or sub-maximal concentrations of NGF, as shown in Fig. 10. In Fig. 10 open squares indicate NGF, diamonds indicate NGF + 25nM IGF-I, open circles represent 25nM IGF-I, and the horizontal line at 554 DPM represents uninduced cells. The percent increases in ChAT activity over control uninduced cells are summarized in Table 7.

Cultured rat septal cell experiments were performed generally as described in Hartikku and Hefti, J. Neuroscience, 8:2967-2985 (1985), Hayashi and Patel, Dev. Brain Res., 36:109-120 (1987), and as follows.

Table 7

ADDITIVE EFFECTS OF NGF AND IGF-I
ON CHAT ACTIVITY IN CULTURED RATE SEPTAL CELLS

Growth Factor	Concentration nM	% increase over control
NGF	2.0	44
IGF-I	1.3	20
	12.5	50
	25.0	37
2 nM NGF + IGF-I	1.3	58
	12.5	93
	25.0	100
IGF-I		44
NGF	0.02	56
	0.2	36
	2.0	44
25 nM IGF-I + NGF	0.02	75
	0.2	100
	2.0	94

Dissociated cell cultures of the septal region of day 17 embryonic rats were prepared by standard techniques known to those skilled in the art, using enzymatic (Dispase, Collaborative Research) dissociation of tissue. Cells wereseeded (plated) at 6 x 10^5 cells/cm^2 in poly-l-ornithine-laminin coated plastic tissue culture wells, and cultured in serum-free N2 medium (Bottenstein et al., 1978) for 5 days without feeding. Control (uninduced) cultures received no added growth factors; induced cultures received the concentrations of IGF-I and NGF indicated in Figs. 9 and 10 at the time of plating. NGF is commercially available. ChAT was assayed by the method described in McManaman, et al. Dev. Biol. 125:311-320 (1988). AChE staining was performed according to the method of Hartikka and Hefti, J. Neuroscience 8:2967-2985 (1988).

Positive cytochemical staining for the enzyme acetylcholinesterase (AChE) has been shown to be a reliable marker for choline acetyltransferase positive neurons in rat septal cell cultures (Hartikka and Hefti, J. Neuroscience 8:2967-2985 (1988).

EXAMPLE 8

The sequence in which NGF and IGF-I are added to the culture medium has a significant effect on the magnitude of the increase of ChAT activity in cultured rat septal cells, as shown in Fig. 11. In Fig. 11, A represents 2nM IGF, B represents 25nM IGF-I, C represents IGF-I + NGF, both added 5 days before assay, and D IGF-I added at the beginning of the experiment + NGF added on day 3 of the experiment, with assay on day 5 of the experiment. When added separately, NGF or IGF-I increased ChAT activity 50 to 60% in a 5 day old culture. When NGF and IGF-I were present together for the entire 5 days the NGF and IGF-I effects on ChAT activity

were additive (a 100% increase), as shown in Figs. 9, 10, and 11.

When IGF-I was present from the beginning of the experiment and NGF was added on day 3, the ChAT activity on day 5 was increased by 300% over uninduced cultures, as shown in Fig. 11. Thus it has been discovered that IGF-I and NGF act in a previously unknown, complimentary manner to enhance the survival and neurotransmitter-synthesizing capacity of cholinergic neurons.

Cultured rat septal cell experiments were performed as described above.

EXAMPLE 9

We have shown that under specific culture conditions (4 x 10^5 cells/cm^2 in the presence of medium containing 10% bovine calf serum), IGF-I increased the number of AChE positive cells by 3-4 fold over control, growth factor-free cultures, Fig. 12. In Fig. 12 A represents uninduced cells, B represents cells treated with 2nM NGF, C represents cells treated with 100nM IGF-I, and D represents cells treated with NGF + IGF-I. (DPM= radioactive disintegrations per minute.) NGF under the same conditions, did not affect the number of AChE positive cells. These results indicate that IGF-I has a greater effect on cholinergic cell survival (i.e. increases cholinergic survival), while NGF regulates (increases) ChAT activity in existing cholinergic neurons.

EXAMPLE 10

Cationization is a process by which free carboxyl groups of acidic amino acid residues on a polypeptide (i.e., aspartic acid and glutamic acid residues) are modified in order to increase the net positive charge on the polypeptide. The process of cationization has been used to enhance the cellular uptake of large molecules such as albumin and horseradish peroxidase into mouse fibroblast

cells (Shen et al., Proc. Nat. Acad. Sci. USA 75:1872-1876 (1978)). Kumagai et al., J. Biol. Chem. 262:15214-15219 (1987), using intact microvessels from bovine brain that are reportedly a model system for measuring transport across the blood-brain barrier, showed that uptake of cationized albumin by isolated bovine brain microvessels was enhanced when compared with uptake of native albumin.

For global modification of free carboxyl groups, the polypeptide (e.g., NGF, IGF-I, IGF-II or a functional derivative) would be reacted with excess hexamethylenediamine (HMD) (15.5 g/g total protein) for 30 minutes at room temperature, followed by covalent coupling of HMD with 1-ethyl-3[-3-dimethyl- aminopropyl] carbodiimide hydrochloride (EDAC) (1.0g/g total protein) for 3 hours at room temperature. Unreacted species may be removed by filtration using Centricon-3 MPS-1 separation devices (Amicon, Danvers, MA) or ion exchange chromatography. The purified polypeptide may be analyzed using isoelectric focusing to determine the amount of cationization.

If the global modification is used on a polypeptide that is a ligand which binds to a cell surface receptor, and the modification produces a molecule lacking biological activity, the cationization process may be repeated as described above except that the polypeptide would be pre-bound to an appropriate receptor prior to cationization, in order to protect the receptor-binding site on the polypeptide. This protection procedure would be carried out as follows: Tissue, e.g., brain, containing receptors for the polypeptide of interest (e.g., IGF-I) is prepared as described above in Example 1. After incubation with the polypeptide ligand for 2 hours at 4°C to permit receptor binding, the reaction mixture is brought to room temperature, and the cationization procedure carried out

using HMD and EDAC as described above. The reaction mixture is then centrifuged at 16,000 rpm at 4°C for 30 sec in an SS-34 rotor in a Sorvall RC5B centrifuge. The supernatant is discarded and the pellet washed three times in PBS with bovine serum albumin (1 mg/ml). The pellet is resuspended in 100mM acetic acid and incubated for 10 min at 4°C to release the cationized polypeptide from its receptors. After centrifugation again at 16,000 rpm, the supernatant, which contains the released cationized polypeptide, is pH-neutralized with NaOH. It may then be analyzed by isoelectric focusing, by a receptor-binding assay as described in Example 1, or by any appropriate assay for biological activity.

EXAMPLE 11

An alternative to the global modification method is to couple polylysine to at least one free carboxyl group on a polypeptide (such as IGF-I, IGF-II, or a functional derivative of either) with or without receptor protection as described above in Example 10. The procedure follows the method of Shen et al., 1978. For example, polylysine, IGF-I and carbodiimide are added in a 1:1:1 ratio in water or buffer for 3 hours at room temperature. The modified protein would be separated and analyzed as described above in Example 10.

EXAMPLE 12

A third method for modifying protein carboxyl groups to enhance blood brain barrier transport is to form esters with diazomethane or N,N-dimethylformamide R acetals (DMF acetals), where R is dimethyl, diethyl, dibutyl, dibenzyl, etc. This type of modification rapidly forms esters from negatively charged carboxylic acid groups, thus increasing the overall positive charge. An additional benefit from this modification is that these added ester groups may be

such that they increase the overall lipophilicity of the polypeptide and may be removed by intrinsic esterases in vivo to yield intact growth factor. The procedure for this modification, with or without receptor protection as described above in Example 10, is to react diazomethane or DMF acetals with the polypeptide in a 1:1 ratio in solution for 30 min. at room temperature, followed by purification and characterization as described above in Example 10.

EXAMPLE 13

A fourth method of cationization, with or without receptor protection as described above in Example 10, combines the advantages of polylysine cationization with the formation of cleavable esters to enhance blood-brain barrier transport, as well as to yield intact growth factor following transport. Polylysine may be made reactive by reaction with benzyloxylacetyl chloride followed byhydrogenation and mild esterification procedures (Hassner et al., Tet. Let. 46:4475-4478 (1978); Mihara et al., Int. J. Peptide Protein Res. 28:141-145 (1986)). Alternatively, DMF acetal derivatives capable of reacting with polylysine could be used to link polylysine to free carboxyl groups using ester linkages.

EXAMPLE 14

A further type of polypeptide modification is glycosylation: the introduction of glucose or similar residues by reductive amination using, for example, glucose and sodium cyanoborohydride ($NaCNBH_3$). Glycosylation of proteins has been shown to enhance the cellular uptake of these proteins and may prove useful for improving blood-brain barrier transport (Smith et al., Pharm. Res., in press). The procedure for glycosylation, with or without receptor protection as described above in Example 10, is based on the method of Schwartz et al., 1977, wherein a

polypeptide such as IGF-I, IGF-II, or a functional derivative of either is combined with glucose and $NaCNBH_3$ in a molar ratio of 1:300:1600 in 200 mM phosphate buffer at pH 7 for at least 24 hr at 37 C°. Unreacted entities may be removed as described in Example 10, or with lectin affinity chromatography. In previous studies using glycosylated albumin, the modified albumin was taken up by rat epididymal microvessels at a greater rate than was native albumin (Williams et al., Proc. Nat. Acad. Sci. USA 78:2393-2397 (1981)).

EXAMPLE 15

Blood-Brain Barrier Transport Model: Method of Audus et al., Ann.N.Y.Acad.Sci. 507:9-18 (1987).

Microvessel endothelial cells are isolated from the cerebral gray matter of fresh bovine brains. Brains are obtained from a local slaughter house and transported to the laboratory in ice cold minimum essential medium (MEM) with antibiotics. Under sterile conditions the large surface blood vessels and meninges are removed. The cortical gray matter is removed by aspiration, then minced into < 1 mm cubes. The minced gray matter is then incubated with 0.5% dispase (BMB, Indianapolis, IN) for 3 hours at 37°C in a shaking water bath. Following the 3 hour digestion, the mixture is concentrated by centrifugation (1000 xg for 10 min.), then resuspended in 13% dextran and centrifuged for 10 min. at 5800 xg. Supernatant fat, cell debris and myelin are discarded and the crude microvessel pellet is resuspended in 1mg/ml collagenase/dispase and incubated in a shaking water bath for 5 hours at 37°C. After the 5-hour digestion, the microvessel suspension is applied to a pre-established 50% Percoll gradient and centrifuged for 10 min at 1000 xg. The band containing purified endothelial cells (second band from the top of the gradient) is removed and

washed two times with culture medium (50% MEM/50% F-12 nutrient mix). The cells are frozen (-80°C) in medium containing 20% DMSO and 10% horse serum for later use.

After isolation, approximately 5 X 10^5 cells/cm^2 are plated on culture dishes or 5-12 @m pore size polycarbonate filters that are coated with rat collagen and fibronectin. 10-12 days after seeding the cells, cell monolayers are inspected for confluency by microscopy.

Characterization of the morphological, histochemical and biochemical properties of these cells has shown that these cells possess many of the salient features of the blood-brain barrier. These features include: tight intercellular junctions, lack of membrane fenestrations, low levels of pinocytotic activity, and the presence of gamma-glutamyl transpeptidase, alkaline phosphatase, and Factor VIII antigen activities.

The cultured cells can be used in a wide variety of experiments where a model for polarized binding or transport is required. By plating the cells in multi-well plates, receptor and non-receptor binding of both large and small molecules can be conducted. In order to conduct transendothelial cell flux measurements, the cells are grown on porous polycarbonate membrane filters (Nucleopore, Pleasanton, CA). Large pore size filters (5-12 μm) are used to avoid the possibility of the filter's becoming the rate-limiting barrier to molecular flux. The use of these large-pore filters does not permit cell growth under the filter and allows visual inspection of the cell monolayer.

Once the cells reach confluency, they are placed in a side-by-side diffusion cell apparatus (Crown Glass, Sommerville, NJ). For flux measurements, the donor chamber of the diffusion cell is pulsed with a test substance, then at various times following the pulse, an aliquot is removed

from the receiver chamber for analysis. Radioactive or fluorescently-labelled substances permit reliable quantitation of molecular flux. Monolayer integrity is simultaneously measured by the addition of a non-transportable testsubstance such as sucrose or insulin and replicates of at least 4 determinations are measured in order to ensure statistical significance.

EXAMPLE 16

To determine whether carboxy-terminal linear peptide derivatives of IGFs can promote the survival of cortical neuronal cells, dissociated cultures of embryonic rat cortex were prepared and assayed for the total number of viable cells present after incubation in the presence or absence of peptide. Cortices were dissected from E18 embryonic rats, dissociated by enzymatic digestion or mechanical dissociation and seeded at a density of 6.25×10^4 cells/cm^2 in defined insulin/serum-free media (Bottenstein and Sato. PNAS, 76:514-517,(1979)) in the presence or absence of 100 μM peptide. The total number of cells remaining after 4 days was assayed by incubation with the vital stain calcein-AM at 6 μM. This compound is taken up by all cells but can only be converted to a fluorescent derivative by live cells. The relative fluorescence values obtained reflect the total viable cell number. The relationship between cell number and relative fluorescence is linear (Figure 13), indicating that this is a useful assay to measure relative differences in cell numbers. The carboxy-terminal linear peptide derivatives analyzed were derived from the amino acid regions 55-70 of IGF-I and comparable regions of IGF-III (SEQ ID NO:4) and the comparable region within IGF-II, amino acids 54-67 (SEQ ID NO:3). We found that peptides IGF-II(54-67) (SEQ ID NO:3), IGF-II(58-67) (SEQ ID NO:2) (Example 19), IGF-I(55-70) (SEQ ID NO:4) (Example 21), IGF-

II(54-67; D-Y) (SEQ ID NO:45) (Example 24), IGF-II(58-67; D-Y) (SEQ ID NO:46) (Example 23), the amino acid sequence EPYCAPPAKSE (SEQ ID NO:5) (Example 22), and the amino acid sequence TYCAPAKSE (SEQ ID NO:1, Example 20) increased the total number of cells surviving within dissociated preparations of E18 cortical neuronal cultures relative to control cultures incubated without peptide (Figure 14).

When the same procedure was repeated with a different set of peptides, we found that peptides EALLETYCATPAKSE (SEQ ID NO:36), ALLEKYCAKPAKSE (SEQ ID NO:37), IGF-II (58-65) (SEQ ID NO:51), IGF-II (56-63) (SEQ ID NO:52), IGF-II (60-67) (SEQ ID NO:53), TDYCAPAKSE (SEQ ID NO:50), YCAPAKSE (SEQ ID NO:54), YCAPA (SEQ ID NO:55), TYCAPA (SEQ ID NO:56), CAPAKSE (SEQ ID NO:24), iodinated-TYCAPAKSE (SEQ ID NO:25), and APSTCEYKA (SEQ ID NO:38) (see Tables 3 and 4) also increased the total number of cells surviving within dissociated preparations of E18 cortical neuronal cultures relative to control cultures incubated without peptide (Figure 14a). All of the peptides showing cortical cell survival activity (Figures 14 or 14a) also showed neurite outgrowth as described below (Example 18).

These findings are significant because they define a novel region within the IGFs, and specifically identify novel sequences that possess biological function. The peptides of Examples 22 and 23 are modified peptides containing the D isomer of tyrosine (D-Y) at position 59 within IGF-II amino acids 54-67 or 58-67. These modifications were made to prevent peptide bond degradation due to enzymatic cleavage amino terminal to position 60 of IGF-II(54-67) and (58-67). Using a non-denaturing HPLC size exclusion chromatography technique the integrity of peptides IGF-II(54-67) and IGF-II(58-67) was analyzed before and after incubation with cells. Figure 15 illustrates that the

D-Y modified peptide IGF-II(54-67) (SEQ ID NO:45) is stabilized against degradation when incubated with cells.

EXAMPLE 17

To determine the potency of the carboxy terminal peptides we analyzed the ability of peptides to promote cell survival. We measured the total number of surviving cells within cortical neuronal cultures relative to basal control cultures. Figure 16 illustrates the concentration-response relationship for peptide TYCAPAKSE (SEQ ID NO:1) and demonstrates that this peptide promotes cortical neuronal cell survival in a dose-dependent manner. All peptides examined are effective within the micromolar concentration range in the fluorometric viability assay.

EXAMPLE 18

To determine whether carboxy-terminal linear peptides of IGFs could promote cortical neurite regeneration, dissociated preparations of E18 rat cortices were cultured in defined insulin/serum-free media in the presence or absence of 100 μM peptides: IGF-II(54-67) (SEQ ID NO:3), IGF-I(55-70) (SEQ ID NO:4), peptide TYCAPAKSE (SEQ ID NO:1), peptide EPYCAPPAKSE (SEQ ID NO:5), IGF-II(54-67; D-Y) (SEQ ID NO:45) and IGF-II(58-67) (SEQ ID NO:2). The cultures were observed 96 hours after peptide exposure and photomicrographs were taken. As illustrated in Figure 17, peptide-treated cultures displayed more cells with neurite outgrowth than untreated basal control cultures. Similar neurite regenerative responses were observed with all the peptides of this Example. These results demonstrate that carboxy-terminal linear peptide derivatives of IGFs and a novel amino acid sequence not only have a survival promoting effect but also an axonal regenerative effect on cortical neurons.

EXAMPLE 19

The compound TYCATPAKSE (IGF-II(58-67)) (SEQ ID NO:2) was prepared by the solid phase method of peptide synthesis using a Milligen BioSearch Model 9600 Peptide Synthesizer. The general procedure is that described by Hudson (Hudson, J. Chem., 53:617-624 (1988)).

One gram of Fmoc-Glu(t-Butyl)-p-alkoxybenzyl alcohol resin (Milligen/Biosearch) was placed in the reaction vessel and was sequentially allowed to react with 1.5 mM solutions of

1) Fmoc-L-Serine-t-butyl ether
2) ε-t-butyloxycarbonyl-Fmoc-L-Lysine
3) Fmoc-Alanine
4) Fmoc-Proline
5) Fmoc-Threonine-t-butyl ether
6) Fmoc-Alanine
7) S-triphenylmethyl-Fmoc-Cysteine
8) Fmoc-Tyrosine-t-buytl ether
9) Fmoc-Threonine-t-butyl ether

in 1:1 dimethylformamide (DMF)/dichloromethane (DCM) using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 1-hydroxybenzotriazole (HOBT) as a coupling agent. After each coupling the resin was washed successively with DMF, then DMF/DCM. A solution of 30% of piperidine in 70% of a 1:1 solution of DMF and toluene was used to remove the Fmoc- group, and the resin was washed repeatedly with DMF/DCM. Finally, the crude peptide was removed from 1.41 g of the resin by treatment with 10 mL of a deblocking cocktail containing 90% trifluoroacetic acid, 5% thioanisole, 3% ethanedithiol and 2% anisole. The resulting solution was poured into 10 volumes of ether, and the precipitated solid was separated by filtration and washed with ether.

The resulting crude peptide weighed 0.350 gm. A 100 mg portion was dissolved in 0.5 mL of methanol containing

20μL of 2-mercaptoethanol to suppress dimerization. The solution was injected in 15 portions into a Waters C8 Bondapak preparative column (25x100 mm) on a Hewlett Packard Model 1050 HPLC system using a solvent gradient of 5% to 60% (in 40 min) acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 9.0 ml per min. The eluted solvent was monitored by an ultraviolet detector at 215 nm.

The peak eluting at 10 min. was collected from a series of nine identical injections. The combined eluates were diluted with an equal volume of water and lyophilized to give 25 mg of purified TYCATPAKSE (SEQ ID NO:2).

The identity of the product was verified by fast atom bombardment mass spectrometry (FAB-MS).

M+H (Calculated) 1070.32
M+H (Found) 1070.00

Amino acid analysis:

	Calculated	Found
Glutamic acid	1.0	1.10
Serine	1.0	0.92
Threonine	2.0	2.04
Alanine	2.0	1.94
Proline	1.0	0.85
Tyrosine	1.0	0.87
Lysine	1.0	1.14
Cysteine	1.0	(not determined)

Peptide content was calculated as 84.5%

EXAMPLE 20

By a procedure similar to that in the preceeding example the peptide IGF-II(58-67 des Thr62) TYCAPAKSE, (SEQ ID NO:1) was prepared from 0.5 g of resin (same as used in example 1) and one mmole of the respective amino acid derivatives. From the resulting 125 mg of crude peptide, 95

mg was dissolved in 0.6 mL water containing 40 µL 2-mercaptoethanol. A Vydac C18 semipreparative column in the same HPLC system using a solvent gradient 5% to 60% (in 40 min) acetonitrile at a flow of 3.5 mL per minute was used. From a series of 10 identical injections the peak eluting at 9.5 min was collected and lyophilized to give 24.5 mg of the peptide, TYCAPAKSE (SEQ ID NO:1).

The identity of the product was verified by fast atom bombardment mass spectrometry (FAB-MS).

M+H (calculated) 969.19
M+H (found) 969.00

Amino acid analysis:

	Calculated	Found
Glutamic acid	1.0	1.07
Serine	1.0	1.01
Threonine	1.0	1.07
Alanine	2.0	2.21
Proline	1.0	1.08
Tyrosine	1.0	1.00
Lysine	1.0	0.82
Cysteine	1.0	(not determined)

Peptide content was calculated as 82.0%

EXAMPLE 21

The peptide R R L E M Y C A P L K P A K S A (IGF I(55-70)(SEQ ID NO:4)) was prepared by the procedure described in the Example 19 from 0.5 gm of Fmoc-Ala-p-alkoxybenzyl resin (Milligen/Biosearch) and one mole of the respective amino acid derivatives. From the resulting 210 mg of crude peptide, 100 mg was used for purification in a solvent gradient of 15% to 25% (in 30 min) acetonitrile. The conditions and HPLC systems are the same as in example 2. The peak eluting at 14.5 min was collected to give 21 mg of the peptide RRLEMYCAPLKPAKSA (SEQ ID NO:4).

The identity of the product was verified by fast atom bombardment mass spectrometry (FAB-MS).

M+H	(calculated)	1834.47
M+H	(found)	1834.00

Amino acid analysis:

	Calculated	Found
Glutamic acid	1.0	1.13
Serine	1.0	0.89
Arginine	2.0	2.27
Alanine	3.0	2.88
Proline	2.0	1.75
Tyrosine	1.0	0.90
Methionine	1.0	0.83
Leucine	2.0	2.03
Lysine	2.0	2.17
Cysteine	1.0	(not determined)

Peptide content was calculated 71.4%

EXAMPLE 22

By a procedure similar to that in Example 19 the peptide EPYCAPPAKSE (IGF II(57-67 with Pro at 58 and 62)) (SEQ ID NO:5) was prepared from 1.0 gram of the same resin and one mmole of the respective amino acid derivatives. From the resulting 110 mg of the crude peptide 80 mg was used for purification in a solvent gradient of 10% to 30% (in 40 min) acetonitrile. The conditions and HPLC systems are the same as in example 1. The peak eluting at 10.7 min was collected to give 8.3 mg of the peptide, EPYCAPPAKSE (SEQ ID NO:5).

The identity of the product was verified by fast atom bombardment mass spectrometry (FAB-MS).

M+H	(calculated)	1191.33
M+H	(found)	1192.00

Amino acid analysis:

	Calculated	Found

Glutamic acid	2.0	2.14
Serine	1.0	0.78
Alanine	2.0	2.13
Proline	3.0	3.01
Tyrosine	1.0	0.97
Lysine	1.0	1.09
Cysteine	1.0	(not determined)

Peptide content was calculated as 73.3%

EXAMPLE 23

The peptide TDYCATPAKSE (IGF II(58-67 with D-Tyr at 59) (SEQ ID NO:46)) was prepared by the procedure described in Example 19 from 1.0 gm of the same resin. Crude peptide (60 mg) was purified in a solvent gradient 5% to 30% (in 40 min) acetonitrile with the same conditions as in Example 2. The peak eluting at 17.3 min was collected to give 6.5 mg of the peptide, TDYCATPAKSE (SEQ ID NO:46).

The identity of the product was verified by fast atom bombardment mass spectrometry (FAB-MS).

M+H (calculated)	1070.32
M+H (found)	1071.00

Amino acid analysis:

	Calculated	Found
Glutamic acid	1.0	1.00
Serine	1.0	0.97
Threonine	2.0	2.02
Alanine	2.0	2.20
Proline	1.0	1.04
Tyrosine	1.0	0.97
Lysine	1.0	1.02
Cysteine	1.0	(not determined)

Peptide content was calculated as 88.0%

EXAMPLE 24

The peptide ALLETDYCATPAKSE (IGF II(54-67 with D-Tyr at 59) (SEQ ID NO:45)) was prepared from 0.43 gm of the

resin from the synthesis in Example 23 was used to prepare this peptide. Crude peptide (100 mg) was purified in a solvent gradient 15% to 25% (in 40 min) acetonitrile in the same HPLC system as in Example 23. The peak eluting at 13.5 min was collected to give 18.9 mg of the peptide, ALLETDYCATPAKSE (SEQ ID NO:45).

The identity of the product was verified by fast atom bombardment mass spectrometry (FAB-MS).

M+H (calculated)	1497.0
M+H (found)	1469.5

Amino acid analysis:

	Calculated	Found
Glutamic acid	2.0	2.08
Serine	1.0	0.94
Threonine	2.0	2.13
Alanine	3.0	3.12
Proline	1.0	0.95
Tyrosine	1.0	0.97
Leucine	2.0	2.08
Lysine	1.0	0.92
Cysteine	1.0	(not determined)

Peptide content was calculated as 93.0%

EXAMPLE 25

By a procedure similar to that of Example 20, the peptide des Thr62-D-Tyr59 IGF-II(58-67), TD-YCAPAKSE may be prepared from 0.5g of the same resin used in Example 1 and one mmole of the respective amino acid derivatives, with the exception that Fmoc-D-Tyrosine-t-butyl ether is substituted for Fmoc-Tyrosine-t-butyl ether. The resulting crude peptide may be purified by HPLC and characterized as TD-YCAPAKSE (SEQ ID NO:50).

EXAMPLE 25-A

Part 1:

Synthesis of CALLETYCATPAKSEC (SEQ ID NO:6)

The compound CALLETYCATPAKSEC (SEQ ID NO:6) was prepared by the solid phase method of peptide synthesis on a Milligen BioSearch Model 9600 Peptide Synthesizer.

0.5 gm (0.46 mM/gm) of Fmoc-Cys (S-triphenylmethyl)-p-alkoxybenzyl alcohol resin (Advanced ChemTech) was placed in the reaction vessel and was sequentially allowed to react with 1.0 mM solutions of

1) Fmoc-Glutamic acid-γ-t-butyl ester
2) Fmoc-Serine-t-butyl ether
3) ϵ-t-butyloxycarbonyl-Fmoc-Lysine
4) Fmoc-Alanine
5) Fmoc-Proline
6) Fmoc-Threonine-t-butyl ether
7) Fmoc-Alanine
8) S-acetamidomethyl-Fmoc-Cysteine
9) Fmoc-Tyrosine-t-butyl ether
10) Fmoc-Threonine-t-butyl ether
11) Fmoc-Glutamic acid-γ-t-butyl ester
12) Fmoc-Leucine
13) Fmoc-Leucine
14) Fmoc-Alanine
15) S-triphenylmethyl-Fmoc-Cysteine

in 1:1 DMF/DCM using BOP and HOBT as a coupling agent (see Example 19). Finally, the crude peptide CALLETYC(Acm)ATPAKSEC (SEQ ID NO:6) was removed from 0.91 gm of the resin by treatment with 10 mL of a deblocking cocktail containing 90% trifluoroacetic acid, 5% thioanisole, 3% ethanedithiol and 2% anisole. After 4.5h of stirring the mixture was filtered and the filtrate was dried

using argon and precipitated using anhydrous ether. The resulting crude peptide weighed 0.34 gm.

Part 2:

Cyclization of CALLETYC(Acm)ATPAKSEC (SEQ ID NO:6)

The crude peptide (0.3 gm) is dissolved in water (1000 mL) and the pH is adjusted to 8.4 with 50% ammonium hydroxide in water. A dilute solution (0.01 N) of potassium ferricyanide is added dropwise until a pale yellow color persists. After stirring for 2 h, the reaction is quenched by adjusting the solution to pH 4.6 with glacial acetic acid. The excess ferro- and ferricyanide ions are removed by passing through an anion-exchange column. The eluent is concentrated to 10 mL and the solution adjusted to pH 4.6. To remove the acetamidomethyl (Acm) protecting group from the internal Cys, a 0.2 M solution (4 mL) of mercury(II)acetate in 1:1 water/acetic acid is added and the reaction mixture is stirred for an hour. The resulting mixture is desalted and purified by HPLC as described above.

Example 25-B

Synthesis of Cyclic TYCAPAKSE (SEQ ID NO:1)

The compound cyclic TYCAPAKSE (SEQ ID NO:1) was prepared by utilizing solid phase (Milligen BioSearch Model 9600 Peptide Synthesizer) and solution phase methods.

0.79 gram (0.97 mM/gm) of p-alkoxybenzyl alcohol resin (Bachem BioScience) was placed in the reaction vessel and was sequentially allowed to react with 3.0 mM solutions of

1) Fmoc-Glutamic acid-γ-benzyl ester
2) Fmoc-Serine-O-benzyl ether
3) ε-benzyloxycarbonyl-Fmoc-Lysine
4) Fmoc-Alanine
5) Fmoc-Proline

6) Fmoc-Alanine
7) S-acetamidomethyl-Fmoc-Cysteine
8) Fmoc-Tyrosine-O-benzyl ether
9) Fmoc-Threonine-O-benzyl ether

in 1:1 DMF/DCM using [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate (TBTU) and HOBT as a coupling agent. Each of the coupling steps was carried out as described (Example 19). The crude peptide (0.84 g) was removed from 1.82 grams of the resin by treatment with a deblocking cocktail containing 15 mL of TFA, 10 mL of DCM and 0.5 mL of water.

The peptide was dissolved in 30 mL of DMF and added to a solution of 1000 mL DMF containing 2 mL of N-methylmorpholine and 2.5 mL of diphenylphosphorazide over a period of one hour. The solvent was evaporated after overnight stirring. The crude product was dissolved in ethyl acetate (200 mL), and the solution was washed with 2% citric acid, water and 3% sodium bicarbonate. The peptide obtained after evaporation was hydrogenated for an hour using 10% Pd on activated charcoal using ethyl acetate as the solvent. The Acm group was removed from the peptide using mercury (II) acetate and purified using HPLC as described above.

<u>Treating Disorders By Application of Insulin-Like Growth Factors</u>

<u>EXAMPLE 26</u>

Recombinant human IGF-I, IGF-II, and IGF-III, were obtained from commercial sources as indicated in Table I.

The activity of IGF-I, IGF-II and IGF-III was assayed on dissociated cultures of 14.5 day embryonic rat spinal cord neurons. The spinal cords were removed from embryos and dissociated in 0.05% trypsin in Dulbecco's Phosphate Buffered Saline (DPBS). Dissociated cells were

seeded onto poly-l-ornithine substrates in 96 well microtiter plates at 2×10^5 cells/well in 200 µl of serum-free and insulin-free N2 medium (Bottenstein and Sato, 1979 supra) containing 0.05% bovine serum albumin. After a 48 hr incubation at 37°C in a humidified atmosphere of 5% CO_2, 95% air, cultures were assayed for choline acetyltransferase activity according to a modification (McMananman et al., Dev. Bio., 25:311-320 (1988)) of the technique described by Fonnum (Fonnum, J. Neurochem. 24:405-409 (1975)).

In this assay, IGF-I, IGF-II, and IGF-III were all found to produce a substantial, dose-dependent increase in choline acetyltransferase activity (Fig. 18). Under these serum-free conditions in which IGF binding protein concentration would be expected to be low, IGF-I and IGF-III have similar efficacy profiles, whereas IGF-II is slightly less potent (Fig. 18). IGF-III, which due to the absence of the 3 N-terminal amino acids found in IGF-I (glycine, proline and glutamate), does not bind substantially to IGF binding proteins found in serum, shows greater potency in increasing ChAT compared with IGF-I in serum containing medium (Fig. 19). These results provide further evidence that IGF-I, II, and III dramatically enhance the survival and/or neurotransmitter-synthesizing capability of spinal cord neurons.

EXAMPLE 27

The neuronal survival promoting activity of IGF-I and IGF-II was assayed on fetal rat cortical neuronal cultures. Cerebral cortices were removed from embryonic day 18 fetal rats. Cortices were minced and treated with neutral protease (Dispase, Collaborative Research) to yield dissociated cell preparations. Cells were plated at low density (5×10^3 cells/well) onto poly-L-ornithine and laminin coated 1/2 area 96 well plates in 50 µl of serum and

insulin-free N2 medium containing 0.05% BSA and 50 μM leucine. IGF-I or II were present from time of seeding. Control cultures received no growth factor. Cultures were incubated for 24 hr at 37°C in an atmosphere of 5% CO_2, 95% air. After 24 hrs, [^{3}H]leucine was added in an additional 50 μl of modified N2 medium to a final concentration of 12.5 μCi/ml. Twenty-four hours after the addition of [^{3}H]leucine, cultures were harvested, protein precipitated on filters with 10% trichloroacetic acid, and dried filters counted in a liquid scintillation counter. The incorporation of [^{3}H]leucine into cells is an accurate reflection of cell survival, since incorporated [^{3}H]leucine counts correlated directly with the number of surviving cells as measured by microscopic counts of cells in identical cultures.

IGF-I and IGF-II both promoted the survival of cortical neurons with IGF-I being slightly more potent (Fig. 20).

EXAMPLE 28

In the spinal cord, as well as in other regions of the nervous system, neurons are over-produced during development, and undergo a process of naturally occurring cell death prior to parturitio and ends on E10 (reviewed in Oppenheim, R.W. 1991, *Annu Rev. Neurosci.* 14:453-501). During this period, approximately 50% of the motoneurons degenerate and die. Certain neurotrophic agents applied exogenously during this period prevent or retard this form of neuronal death (e.g., partially purified muscle extracts and ciliary neurotrophic factor reduce motor neuron death in chick embryos (McManaman et al., 1990, *Neuron* 4:891-898; Oppenheim et al. 1991, *Science* 251:1616-1617).

IGF-I, as well as several variants ({des 1-3} IGF-I, IGF-II, and R^3 IGF-I, an analog of IGF-I engineered

molecularly by addition of a 13 amino acid extension peptide at the N-terminus and substitution of an Arg for the Glu at position 3 in the natural IGF-I sequence) partially prevented developmentally regulated motoneuron death in this model. The IGFs were administered daily onto chorioallantoic membrane (5 μg/egg in a volume of 50 μl) from embryonic day E6 to E9 through a small window cut into the shell of the egg. Control embryos received an equal volume of vehicle (phosphate buffered saline) alone. On E10, the embryos were removed, sacrificed and the spinal cords sectioned. Motoneurons (identified morphologically in silver-stained sections) were counted in serial sections from both treated and control embryos. The data represented in Figure 21 demonstrate that applications of IGFs resulted in increased motoneuron survival from 17 to 25% over untreated, control embryos. The differences between effects produced by the different IGFs used were not statistically significant (Student t test $P<0.05$).

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Cephalon, Inc.

(ii) TITLE OF INVENTION: TREATING DISORDERS BY APPLICATION OF INSULIN-LIKE GROWTH FACTORS AND ANALOGS

(iii) NUMBER OF SEQUENCES: 56

(iv) CORRESPONDENCE ADDRESS:

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(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 07/361,595
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(A) APPLICATION NUMBER: 07/869,913
(B) FILING DATE: April 15, 1992

(A) APPLICATION NUMBER: 07/958,903
(B) FILING DATE: October 7, 1992

(viii) ATTORNEY/AGENT INFORMATION:

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(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 542-5070
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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Thr Tyr Cys Ala Pro Ala Lys Ser Glu
 1               5
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Thr Tyr Cys Ala Thr Pro Ala Lys Ser Glu
 1               5                  10
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Leu Leu Glu Thr Tyr Cys Ala Thr Pro Ala Lys Ser Glu
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Arg Arg Leu Glu Met Tyr Cys Ala Pro Leu Lys Pro Ala Lys
1 5 10

Ser Ala
15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Glu Pro Tyr Cys Ala Pro Pro Ala Lys Ser Glu
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Cys Ala Leu Leu Glu Thr Tyr Cys Ala Thr Pro Ala Lys Ser Glu Cys
1               5                   10                  15
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
Cys Thr Tyr Cys Ala Thr Pro Ala Lys Ser Glu Cys
1               5                   10
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
Cys Glu Pro Tyr Cys Ala Pro Pro Ala Lys Ser Glu Cys
1               5                   10
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Cys Thr Tyr Cys Ala Pro Ala Lys Ser Glu Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Cys Thr Tyr Thr Ala Pro Ala Lys Ser Glu Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Cys Ala Leu Leu Glu Thr Tyr Ala Thr Pro Ala Lys Ser Glu Cys
1 5 10 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Cys Arg Arg Leu Glu Met Tyr Cys Ala Pro Leu Lys Pro Ala Lys Ser
1 5 10 15

Ala Cys

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Cys Gly Tyr Gly Ser Ser Ser Arg Arg Ala Pro Gln Thr Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Cys Tyr Phe Asn Lys Pro Thr Gly Tyr Gly Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Cys Tyr Phe Asn Lys Pro Thr Gly Tyr Gly Ser Ser Ser Arg Arg Ala
1 5 10 15

Pro Gln Thr Cys
20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Cys Lys Pro Thr Gly Tyr Gly Ser Ser Ser Arg Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 70
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe
1 5 10 15

Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
20 25 30

```
Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
        35                  40                  45

Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Ala Pro Leu
    50                  55                  60

Lys Pro Ala Lys Ser Ala
65                  70
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
Cys Gly Cys Glu Leu Val Asp Ala Leu Gln Phe Val Cys
1               5                   10
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```
Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys
1               5                   10
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A

(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Cys Cys Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys
1 5 10 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Cys Pro Leu Lys Pro Ala
1 5 10 15

Lys Ser Glu

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Cys Cys Phe Arg Ser Cys
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5
(B) TYPE: amino acid

(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Cys Phe Arg Ser Cys
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Cys Ala Pro Ala Lys Ser Glu
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(ix) OTHER INFORMATION: Xaa represents an iodinated tyrosine.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Thr Xaa Cys Ala Pro Ala Lys Ser Glu
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7
(B) TYPE: amino acid

(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Cys Gly Pro Glu Thr Leu Cys
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Cys Gly Tyr Gly Ser Ser Ser Arg Arg Cys Pro Gln Thr Gly Ile Val
1 5 10 15

Asp Glu Cys

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11

(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Cys Cys Pro Leu Lys Pro Ala Lys Ser Ala Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Cys Asp Leu Arg Arg Leu Glu Met Tyr Ala Pro Leu Lys Pro Ala
1 5 10 15

Lys Ser Ala Cys

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Ala Tyr Arg Pro Ser Glu Thr Leu Cys Gly Gly Glu Leu Val Asp Thr
1 5 10 15

Leu Gln Phe Val Cys Gly Asp Arg Gly Phe Tyr Phe Ser Arg Pro Ala
20 25 30

Ser Arg Val Ser Arg Arg Ser Arg Gly Ile Val Glu Glu Cys Cys Phe

35 40 45

Arg Ser Cys Asp Leu Ala Leu Leu Glu Thr Tyr Cys Ala Thr Pro Ala
50 55 60

Lys Ser Glu
65

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Cys Gly Gly Glu Leu Val Asp Thr Leu Gln Phe Val Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Cys Asp Leu Cys Leu Leu Glu Thr Tyr Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Cys Cys Phe Arg Ser Cys Asp Leu Ala Leu Leu Glu Thr Tyr Cys
1 5 10 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Cys Asp Leu Cys Leu Leu Glu Thr Tyr Cys Ala Thr Pro Ala Lys Ser
1 5 10 15

Glu

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Glu Ala Leu Leu Glu Thr Tyr Cys Ala Thr Pro Ala Lys Ser Glu
1 5 10 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Ala Leu Leu Glu Lys Tyr Cys Ala Lys Pro Ala Lys Ser Glu
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

Ala Pro Ser Thr Cys Glu Tyr Lys Ala
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

Cys Cys Phe Arg Ser Cys Asp Leu Cys Leu Leu Glu Thr Tyr Cys
1 5 10 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

Cys Cys Tyr Arg Pro Ser Glu Thr Leu Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Cys Arg Pro Cys Ser Arg Val Ser Arg Arg Ser Arg Gly Ile Val Glu Glu
1 5 10 15

Cys

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

Cys Gly Asp Arg Gly Phe Tyr Phe Ser Arg Pro Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

Cys Cys Thr Pro Ala Lys Ser Glu Cys
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

Cys Asp Leu Cys Leu Leu Glu Thr Ala Thr Pro Ala Lys Ser Glu Cys
1 5 10 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(ix) OTHER INFORMATION: Xaa represents the D-isomer of tyrosine.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

Ala Leu Leu Glu Thr Xaa Cys Ala Thr Pro Ala Lys Ser Glu
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(ix) OTHER INFORMATION: Xaa represents the D-isomer of tyrosine.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Thr Xaa Cys Ala Thr Pro Ala Lys Ser Glu
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(ix) OTHER INFORMATION: Xaa represents the D-isomer of tyrosine.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

Cys Ala Leu Leu Glu Thr Xaa Cys Ala Thr Pro Ala Lys Ser Glu Cys
1 5 10 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(ix) OTHER INFORMATION: Xaa represents the D-isomer of tyrosine.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

Cys Thr Xaa Cys Ala Thr Pro Ala Lys Ser Glu Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(ix) OTHER INFORMATION: Xaa represents the D-isomer of tyrosine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

Cys Thr Xaa Cys Ala Pro Ala Lys Ser Glu Cys
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(ix) OTHER INFORMATION: Xaa represents the D-isomer of tyrosine.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

Thr Xaa Cys Ala Pro Ala Lys Ser Glu
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

Thr Tyr Cys Ala Thr Pro Ala Lys
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

Leu Glu Thr Tyr Cys Ala Thr Pro
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

Cys Ala Thr Pro Ala Lys Ser Glu
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 54:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

Tyr Cys Ala Pro Ala Lys Ser Glu
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 55:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

Tyr Cys Ala Pro Ala

1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 56:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

Thr Tyr Cys Ala Pro Ala
1 5

Claims

1. A substantially pure peptide comprising the amino acid sequence of IGF-II(58-67) (SEQ ID NO:2).

2. The peptide of claim 1, wherein tyrosine-59 of said peptide is a D-amino tyrosine (SEQ ID NO:46).

3. A substantially pure peptide comprising the amino acid sequence TYCAPAKSE (SEQ ID NO:1).

4. The substantially pure peptide of claim 3, wherein said peptide is cyclized by formation of an amide bond.

5. The peptide of claim 3 wherein tyrosine-59 of said peptide is a D-amino tyrosine (SEQ ID NO:50).

6. A substantially pure peptide comprising the peptide IGF-II(54-67;D-Y)(SEQ ID NO:45), wherein tyrosine-59 of said peptide is a D-isomer of tyrosine.

7. A therapeutic composition comprising the analog of a fragment of insulin-like growth factor-II IGF-II(58-67;D-Y) (SEQ ID NO:46) and a pharmaceutically acceptable diluent.

8. Insulin-like growth factor II analog IGF-II(58-67;D-Y) (SEQ ID NO:46) for use in enhancing the survival of neuronal cells in a mammal that are at risk of dying, or for use in treating a head or spinal cord injury of a mammal, for use in enhancing neurite regeneration in a mammal, or for use in treating a disease condition affecting neuronal cells of a mammal, said disease condition comprising stroke, epilepsy, age-related neuronal loss, amyotrophic lateral sclerosis or Parkinson's disease.

9. A therapeutic composition comprising insulin-like growth factor-I derivative IGF-I(55-70) (SEQ ID NO:4) and a pharmaceutically acceptable diluent.

10. Insulin-like growth factor-I derivative IGF-I(55-70) (SEQ ID NO:4) for use in enhancing the survival of neuronal cells in a mammal that are at risk of dying, or for use in treating a head or spinal cord injury of a mammal, for use in enhancing neurite regeneration in a mammal, or for use in treating a disease condition affecting neuronal cells of a mammal, said disease condition comprising stroke, epilepsy, age-related neuronal loss, amyotrophic lateral sclerosis or Parkinson's disease.

11. A substantially pure peptide comprising the amino acid sequence of IGF-I(55-70), RRLEMYCAPLKPAKSA (SEQ ID NO:4).

12. A substantially pure peptide comprising the amino acid sequence EPYCAPPAKSE (SEQ ID NO:5).

13. A peptide of any one of claims 1-6, 11 or 12 for use in therapy.

14. Use of a peptide of any one of claims 1-6, 11, or 12 to enhance the survival of neuronal cells in a mammal that are at risk of dying, or to treat a head or spinal cord injury of a mammal, or to enhance neurite regeneration in a mammal, or to treat a disease condition affecting neuronal cells of a mammal selected from stroke, epilepsy, age-related neuronal loss, amyotrophic lateral sclerosis, and Parkinson's disease.

15. A substantially pure peptide wherein said peptide comprises a sequence selected from the group consisting of

the amino acid sequence CALLETYCATPAKSEC (SEQ ID NO:6), the amino acid sequence CTYCATPAKSEC (SEQ ID NO:7), the amino acid sequence CEPYCAPPAKSEC (SEQ ID NO:8), and the amino acid sequence CTYCAPAKSEC (SEQ ID NO:9), wherein the N-terminal cysteine is connected to the C-terminal cysteine by a covalent bond.

16. A substantially pure peptide, wherein said peptide comprises a sequence selected from the group consisting of the amino acid sequence CALLETᴅYCATPAKSEC (SEQ ID NO:47), the amino acid sequence CTᴅYCATPAKSEC (SEQ ID NO:48), and the amino acid sequence CTᴅYCAPAKSEC (SEQ ID NO:49), wherein the N-terminal cysteine is connected to the C-terminal cysteine by a covalent bond.

17. A substantially pure peptide, wherein said peptide comprises a sequence selected from the group consisting of the amino acid sequence CTYTAPAKSEC (SEQ ID NO:10), the amino acid sequence CALLETYATPAKSEC (SEQ ID NO:11), the amino acid sequence CRRLEMYCAPLKPAKSAC (SEQ ID NO:12), the amino acid sequence CGYGSSSRRAPQTC (SEQ ID NO:13), the amino acid sequence CYFNKPTGYGC (SEQ ID NO:14), the amino acid sequence CYFNKPTGYGSSSRRAPQTC (SEQ ID NO:15), and the amino acid sequence CKPTGYGSSSRC (SEQ ID NO:16), wherein the N-terminal cysteine is connected to the C-terminal cysteine by a covalent bond.

18. A substantially pure peptide, wherein said peptide is selected from the group consisting of the amino acid sequence CDLRRLEMYC (SEQ ID NO:19), the amino acid sequence CCFRSCDLRRLEMYC (SEQ ID NO:20), the amino acid sequence CCFRSC (SEQ ID NO:22), and the amino acid sequence CFRSC (SEQ ID NO:23), wherein said peptide is cyclized by a covalent bond between two residues of said peptide.

19. A substantially pure cyclized peptide consisting essentially of the amino acid sequences CGCELVDALQFVC (SEQ ID NO:18) and CCFRSCDLRRLEMYC (SEQ ID NO:20), wherein said cyclized peptide comprises at least one covalent bond between two residues of said cyclized peptide.

20. A substantially pure peptide, wherein said peptide comprises a sequence selected from the group consisting of the amino acid sequence CGCELVDALQFVC (SEQ ID NO:18), the amino acid sequence CDLRRLEMYCCPLKPAKSE (SEQ ID NO:21), the amino acid sequence CGPETLC (SEQ ID NO:26), the amino acid sequence CGYGSSSRRCPQTGIVDEC (SEQ ID NO:27), the amino acid sequence CGDRGFYFNKPTC (SEQ ID NO:28), the amino acid sequence CCPLKPAKSAC (SEQ ID NO:29), and the amino acid sequence CDLRRLEMYAPLKPAKSAC (SEQ ID NO:30), wherein the N-terminal cysteine is connected to the C-terminal cysteine by a covalent bond.

21. A substantially pure peptide, wherein said peptide is selected from the group consisting of the amino acid sequence CGGELVDTLQFVC (SEQ ID NO:32), the amino acid sequence CCFRSCDDLALLETYC (SEQ ID NO:34), wherein said peptide is cyclized by a covalent bond between two residues of said peptide.

22. A substantially pure cyclized peptide consisting essentially of the amino acid sequences CGGELVDTLQFVC (SEQ ID NO:32) and CCFRSCDLCLLETYC (SEQ ID NO:39), wherein said cyclized peptide comprises at least one covalent bond between two residues of said cyclized peptide.

23. A substantially pure peptide, wherein said peptide comprises a sequence selected from the group consisting of the amino acid sequence CDLCLLETYC (SEQ ID NO:33), the amino acid sequence CDLCLLETYCATPAKSE (SEQ ID NO:35), the amino

acid sequence CCYRPSETLC (SEQ ID NO:40), CRPCSRVSRRSRGIVEEC (SEQ ID NO:41), CGDRGFYFSRPC (SEQ ID NO:42), CCTPAKSEC (SEQ ID NO:43), and CDLCLLETATPAKSEC (SEQ ID NO:44), wherein the N-terminal cysteine is connected to the C-terminal cysteine by a covalent bond.

24. A substantially pure peptide, wherein said peptide comprises a sequence selected from the group consisting of the amino acid sequence TYCATPAK (SEQ ID NO:51), LETYCATP (SEQ ID NO:52), CATPAKSE (SEQ ID NO:53), YCAPAKSE (SEQ ID NO:54), YCAPA (SEQ ID NO:55), TYCAPA (SEQ ID NO:56), CAPAKSE (SEQ ID NO:24), EALLETYCATPAKSE (SEQ ID NO:36), ALLEKYCAKPAKSE (SEQ ID NO:37), and APSTCEYKA (SEQ ID NO:38).

25. The peptide of claim 15, 16, 17, 20, or 23, wherein said peptide contains between 5 and 40 amino acids.

26. The peptide of claim 25, wherein said peptide is iodinated.

27. The peptide of claim 25, wherein residues flanking said amino acid sequence are homologous to the naturally occurring sequence of IGF-I, or to the naturally occurring sequence of IGF-II.

28. The peptide of claim 13 or 14, wherein said peptide is a cyclic peptide.

29. The peptide of claim 28, wherein said peptide is iodinated.

30. The peptide of claim 28, wherein said cyclic peptide consists essentially of 5-40 amino acid residues.

31. The peptide of claim 30, wherein said cyclic peptide consists essentially of 6-25 amino acid residues.

32. The peptide of claim 13 or 14, wherein said peptide is a retro-inverso peptide.

33. The peptide of claim 32, wherein said retro-inverso peptide is homologous to IGF-I, or a fragment thereof.

34. The peptide of claim 32, wherein said retro-inverso peptide is homologous to IGF-II, or a fragment thereof.

35. A peptide of any one of claims 15-27 for use in therapy.

36. Use of a peptide of any one of claims 15-27 to enhance the survival of neuronal cells in a mammal that are at risk of dying, or to treat a head or spinal cord injury of a mammal, or to enhance neurite regeneration in a mammal, or to treat a disease condition affecting neuronal cells of a mammal selected from stroke, epilepsy, age-related neuronal loss, amyotrophic lateral sclerosis, and Parkinson's disease.

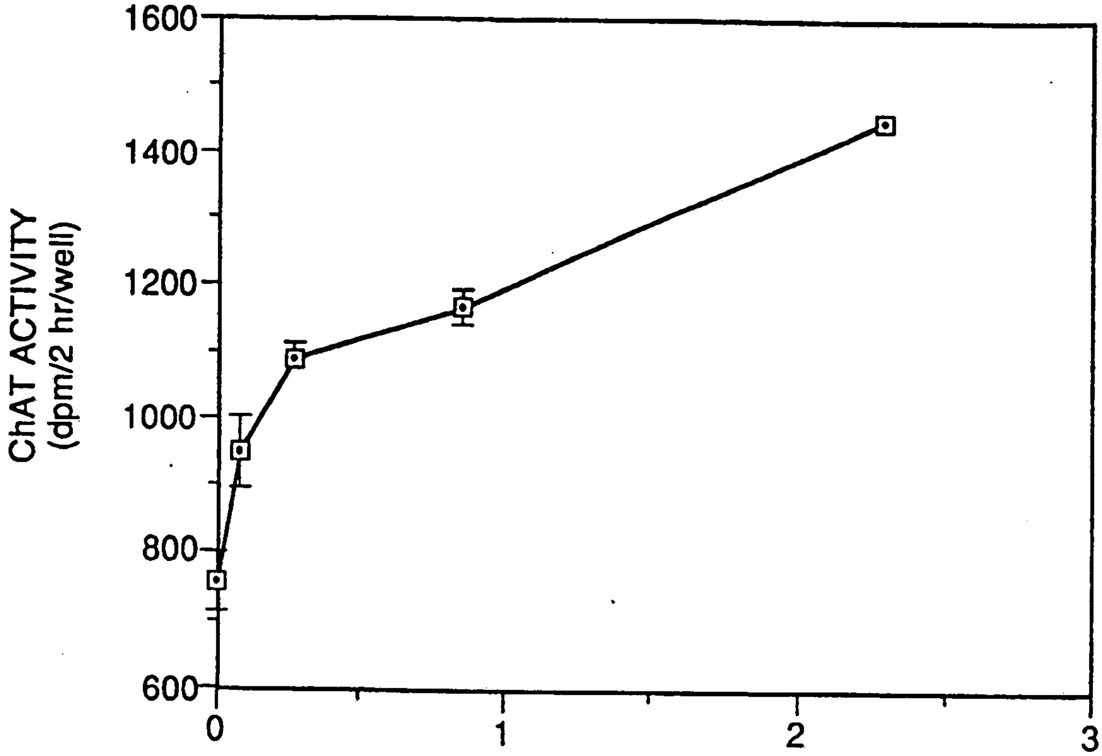

ChAT ACTIVITY
(dpm/2 hr/well)
1600
1400
1200
1000
800
600
0
1
2
3
IGF-I (μM)

FIG 1

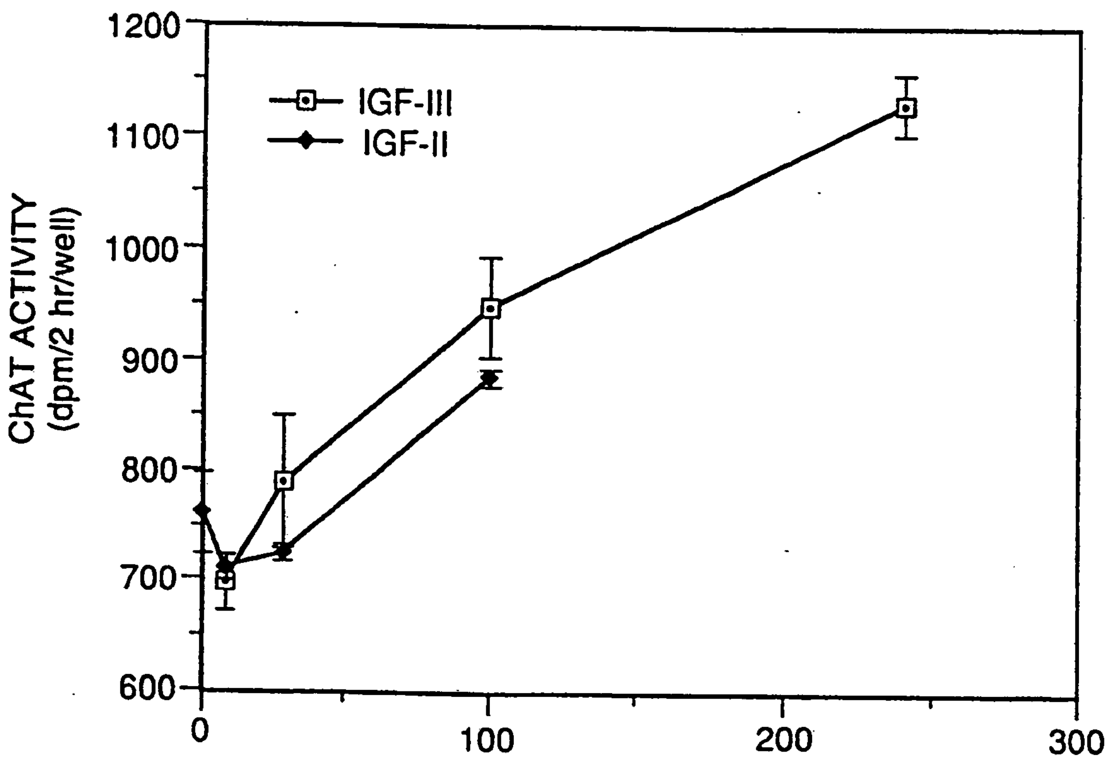

IGF-III
IGF-II
ChAT ACTIVITY
(dpm/2 hr/well)
1200
1100
1000
900
800
700
600
0
100
200
300
IGF (nM)

FIG 2

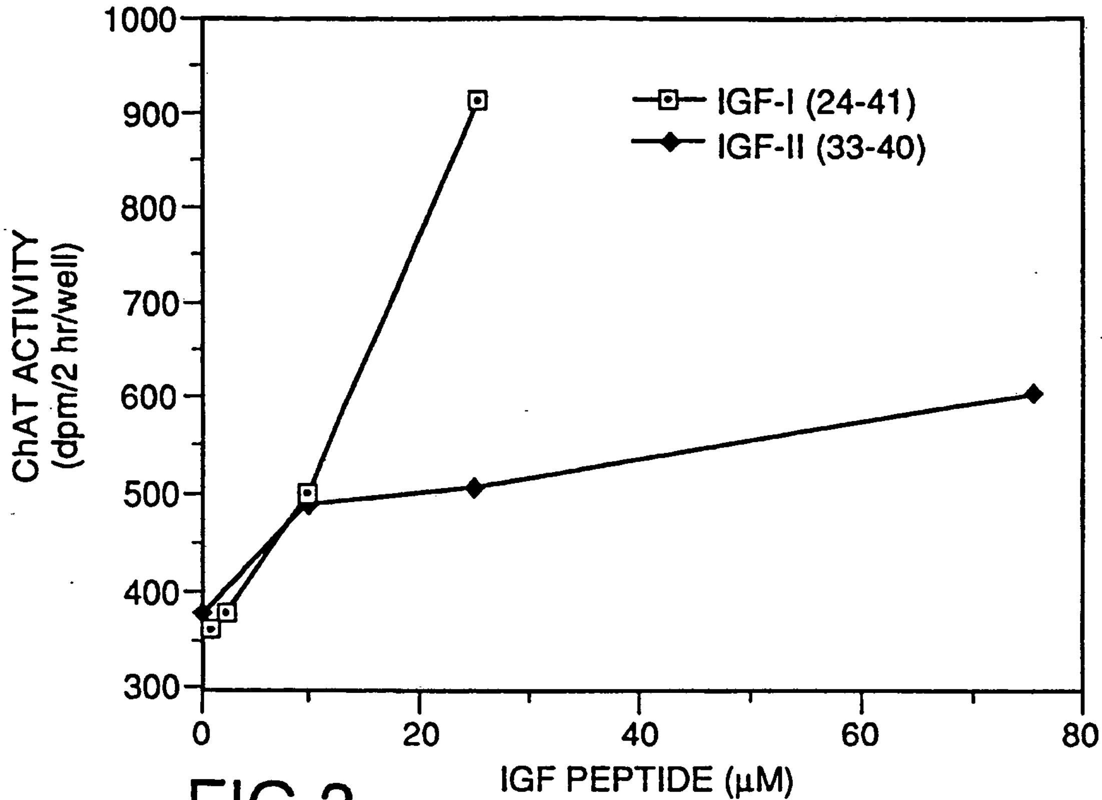

1000
900
800
700
600
500
400
300
ChAT ACTIVITY
(dpm/2 hr/well)
IGF-I (24-41)
IGF-II (33-40)
0
20
40
60
80
IGF PEPTIDE (μM)

FIG 3

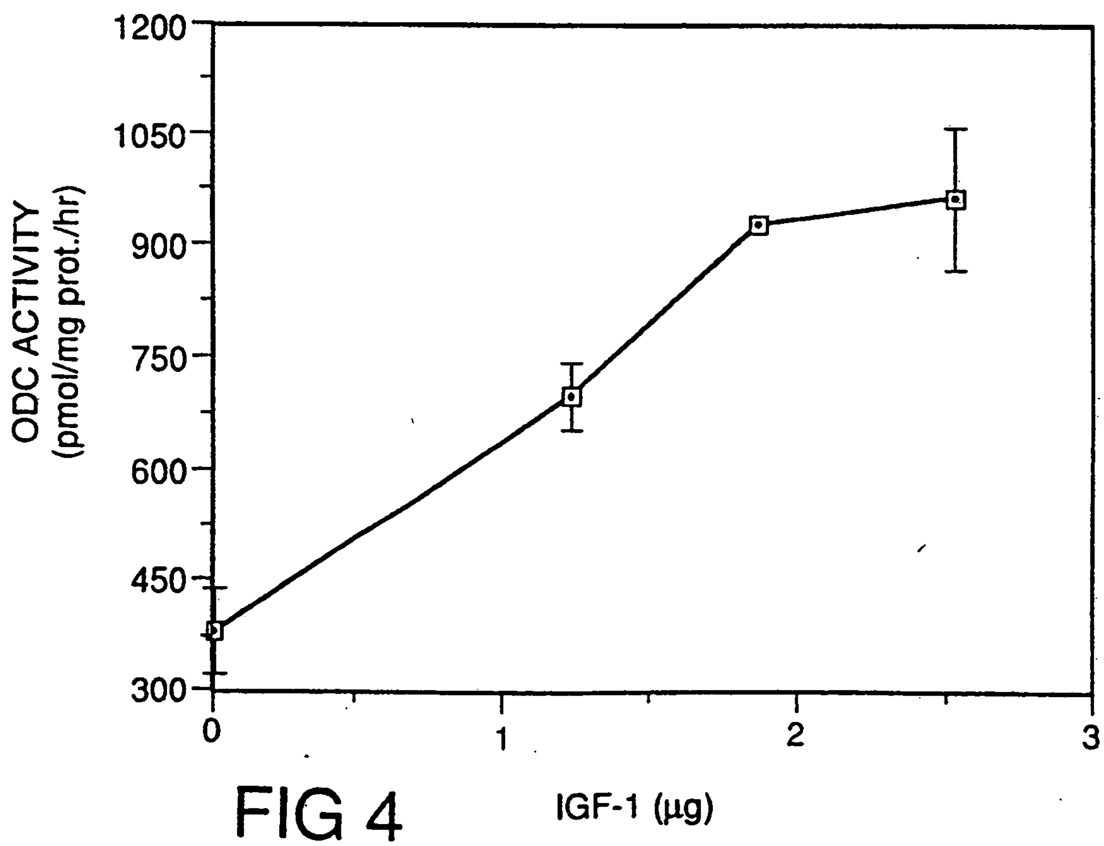

1200
1050
900
750
600
450
300
ODC ACTIVITY
(pmol/mg prot./hr)
0
1
2
3
IGF-1 (μg)

FIG 4

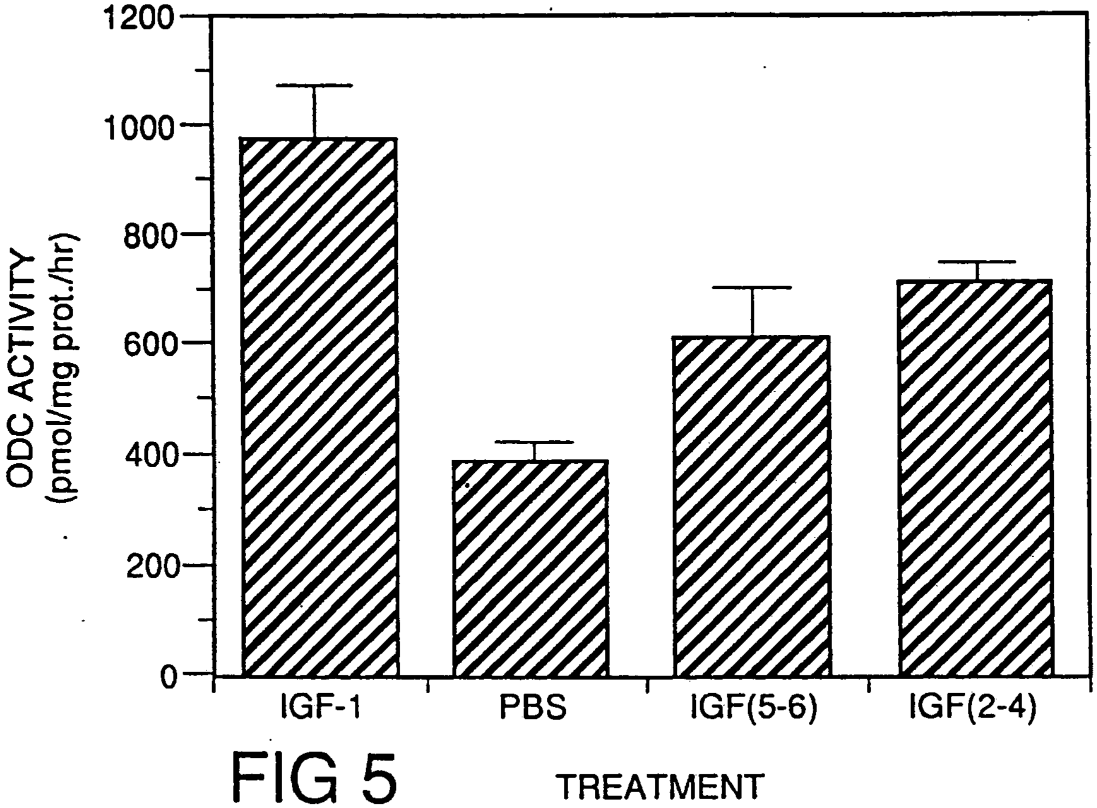

1200
1000
800
600
400
200
0
ODC ACTIVITY
(pmol/mg prot./hr)
IGF-1
PBS
IGF(5-6)
IGF(2-4)
TREATMENT

FIG 5

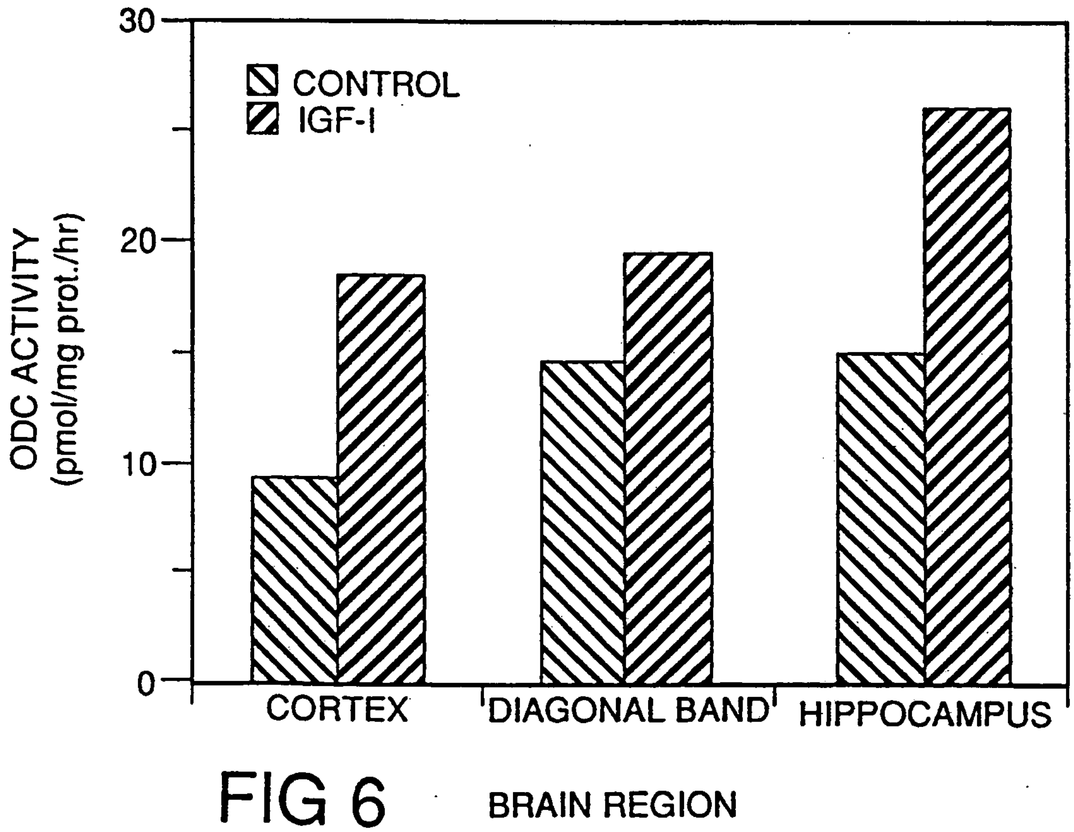

30
20
10
0
CONTROL
IGF-I
ODC ACTIVITY
(pmol/mg prot./hr)
CORTEX
DIAGONAL BAND
HIPPOCAMPUS
BRAIN REGION

FIG 6

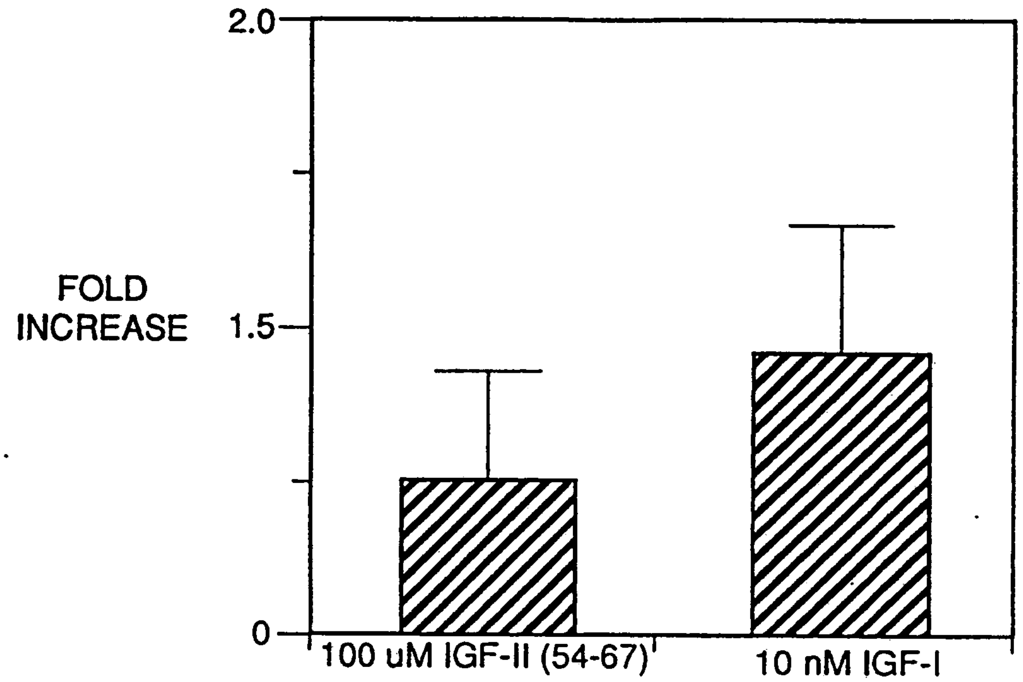

2.0
FOLD
INCREASE
1.5
0
100 uM IGF-II (54-67)
10 nM IGF-I

FIG 7

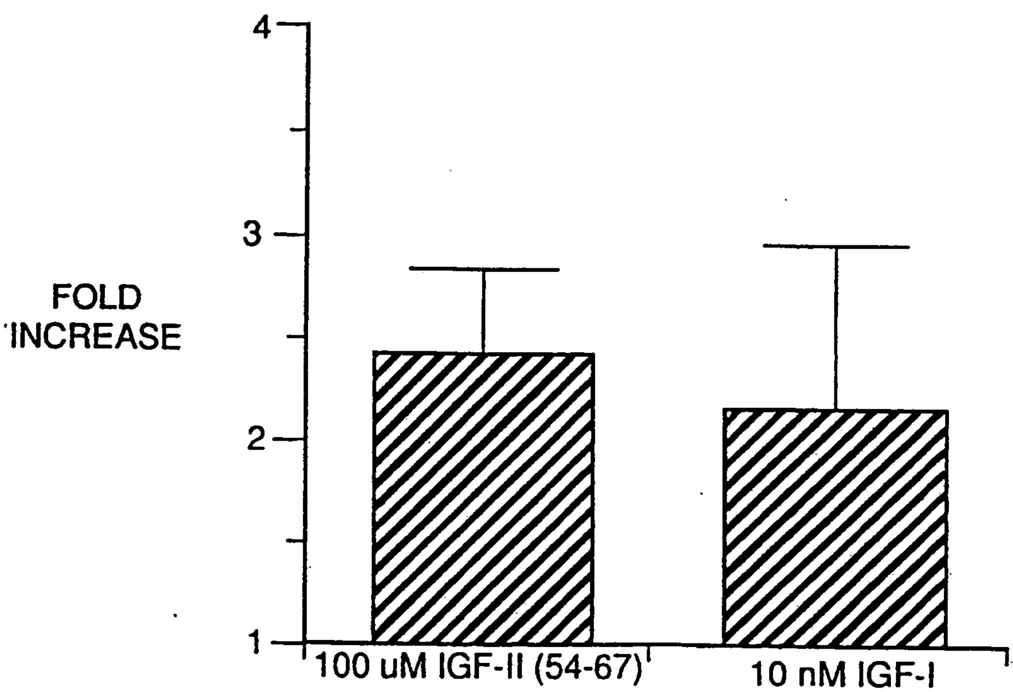

4
3
FOLD
INCREASE
2
1
100 uM IGF-II (54-67)
10 nM IGF-I

FIG 8

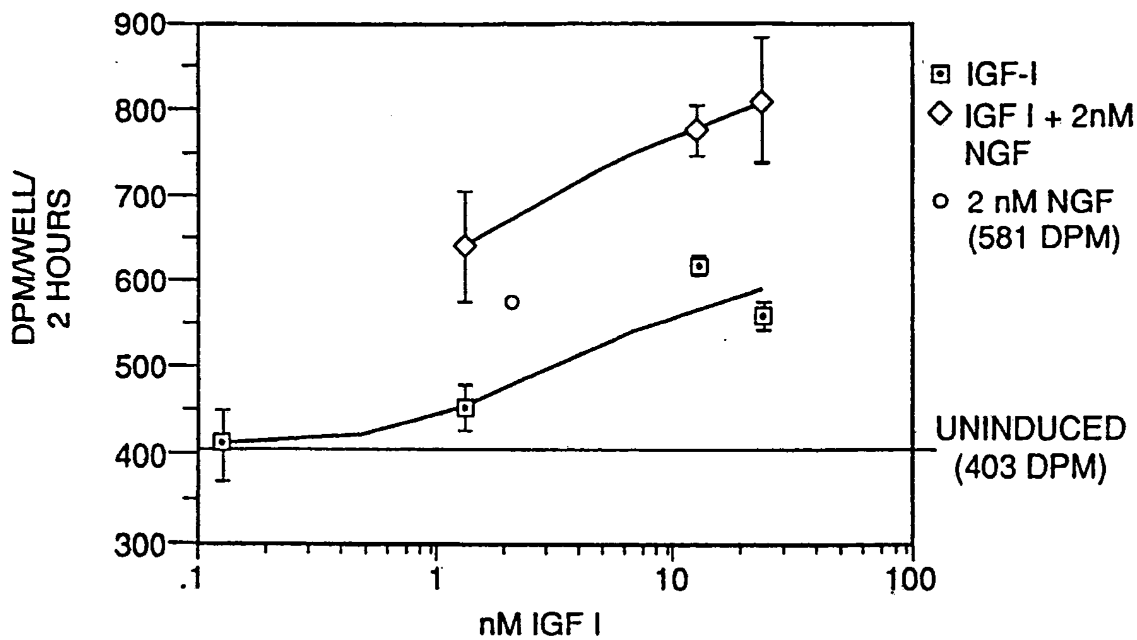

900
800
700
600
500
400
300
DPM/WELL/
2 HOURS
IGF-I
IGF I + 2nM
NGF
2 nM NGF
(581 DPM)
UNINDUCED
(403 DPM)
.1
1
10
100
nM IGF I

FIG 9

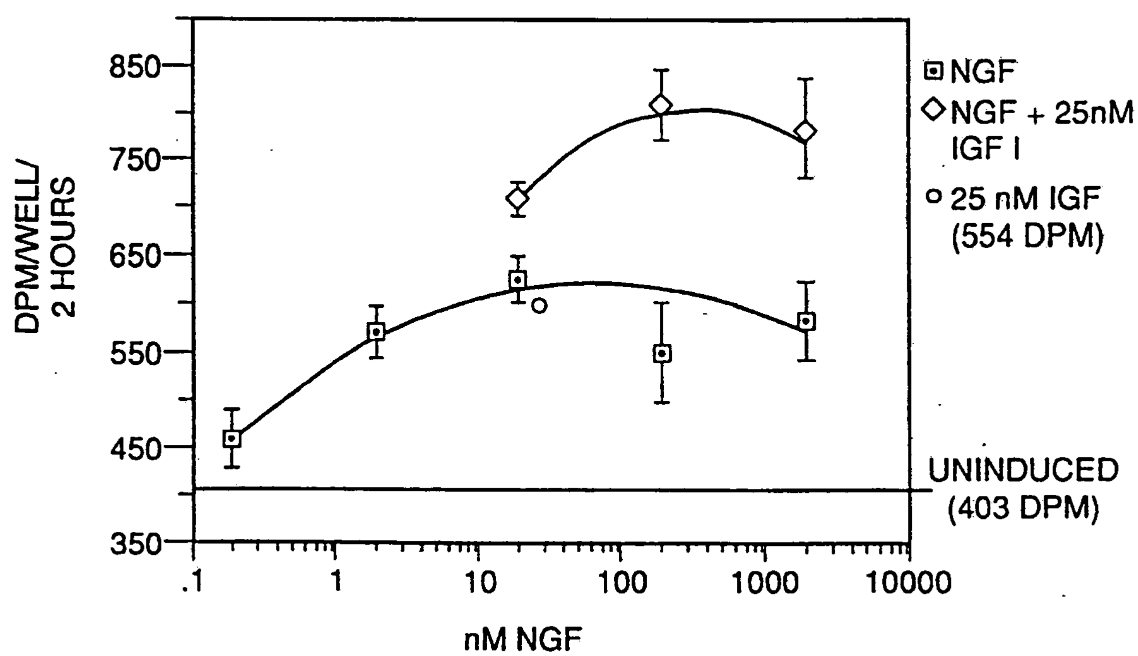
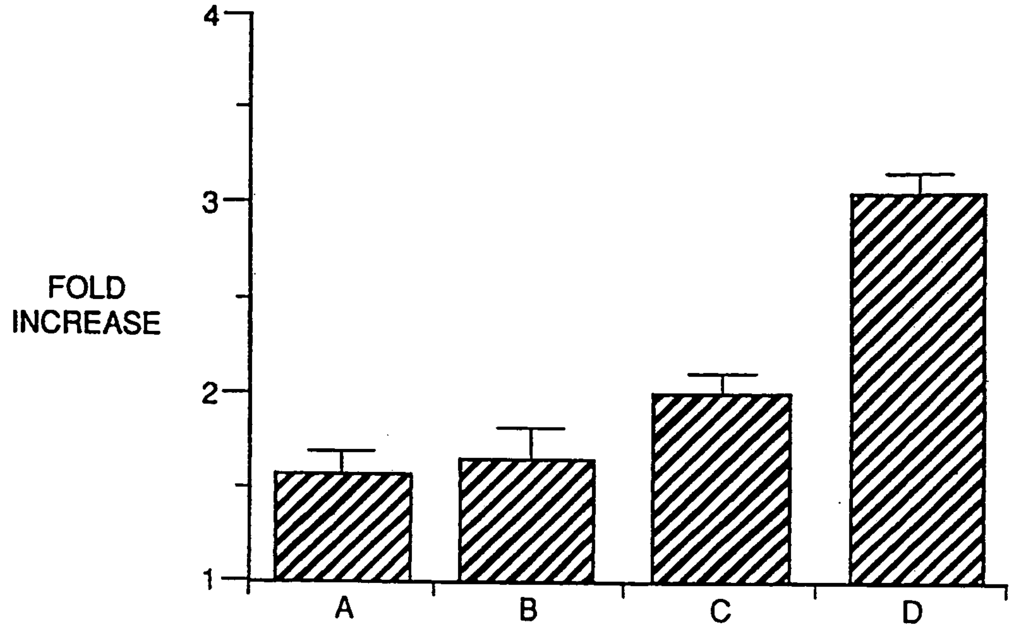

850
750
650
550
450
350
DPM/WELL/
2 HOURS
NGF
NGF + 25nM
IGF I
25 nM IGF
(554 DPM)
UNINDUCED
(403 DPM)
.1
1
10
100
1000
10000
nM NGF

FIG 10



A: 2nM NGF
B: 25 nM IGF I
C: IGF I + NGF, 5 DAYS
D: IGF I, 5 DAYS + NGF
ADDED ON DAY 3

FIG 11

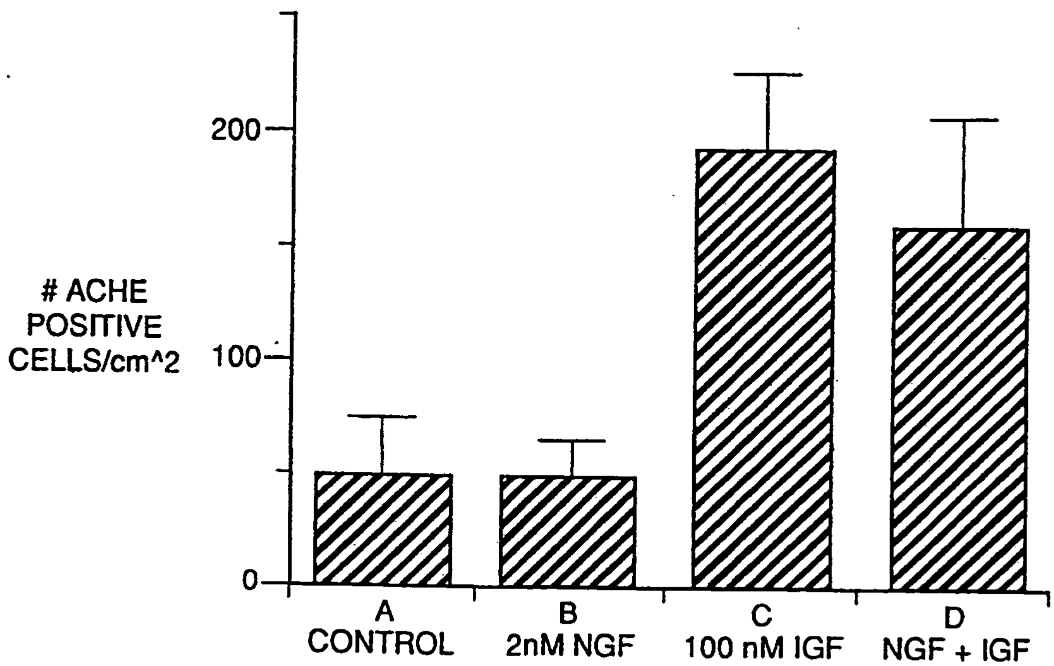


FIG 12

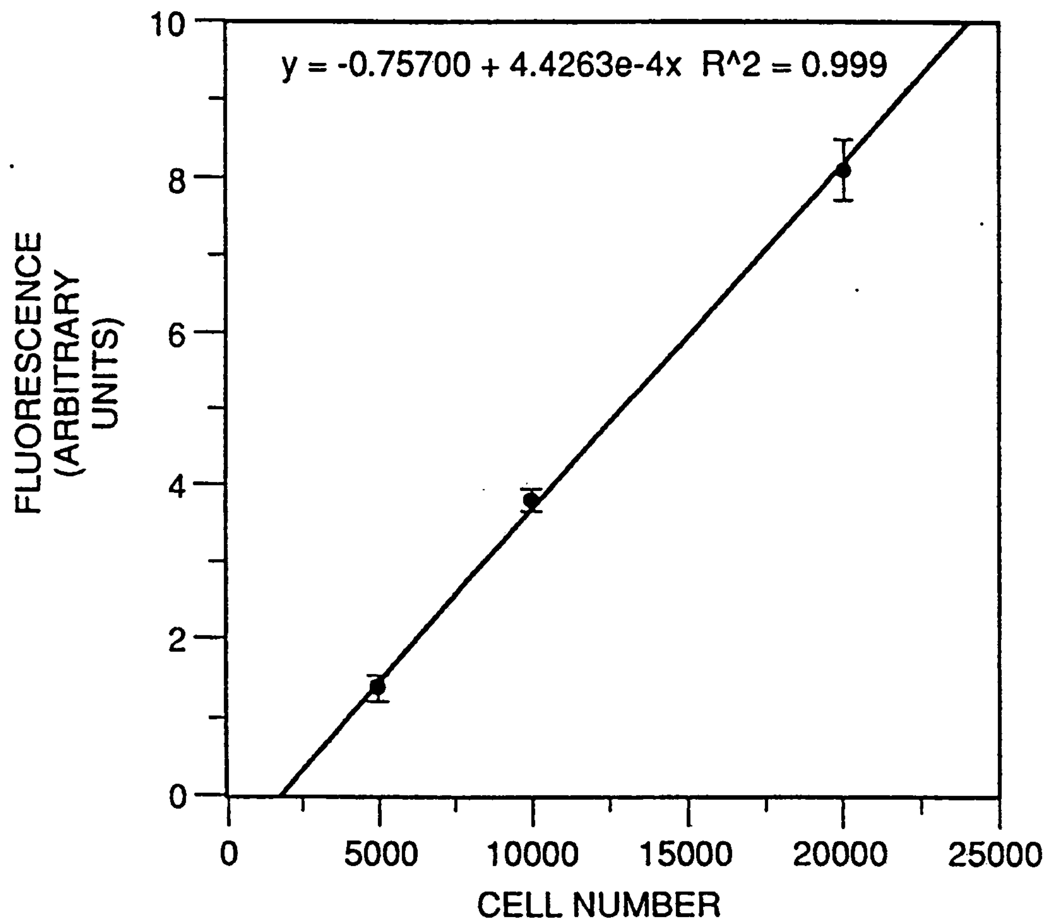
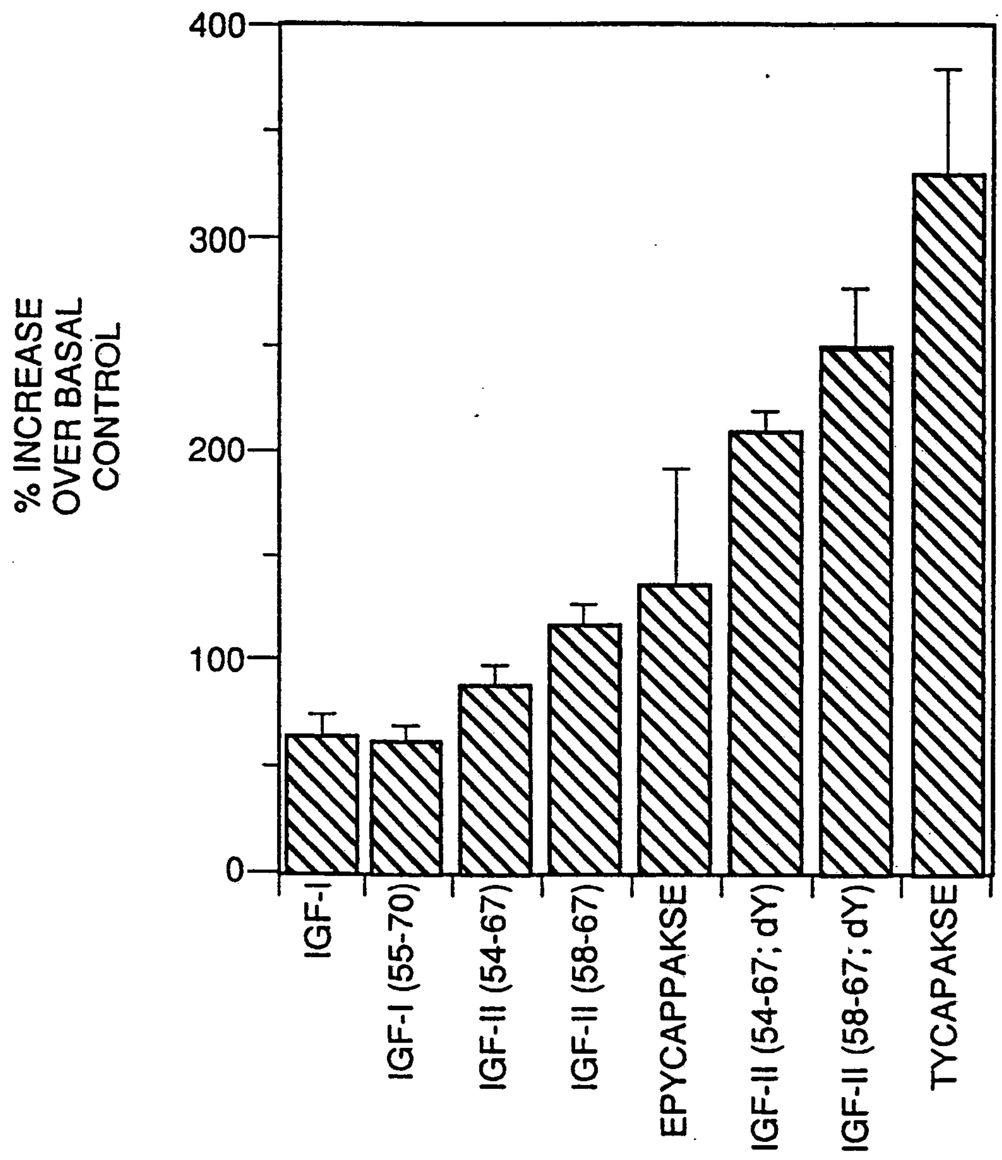

y = -0.75700 + 4.4263e-4x R^2 = 0.999
FLUORESCENCE (ARBITRARY UNITS)
CELL NUMBER
0
2
4
6
8
10
0
5000
10000
15000
20000
25000

FIG 13



400
300
200
100
0
% INCREASE OVER BASAL CONTROL
IGF-I
IGF-I (55-70)
IGF-II (54-67)
IGF-II (58-67)
EPYCAPPAKSE
IGF-II (54-67; dY)
IGF-II (58-67; dY)
TYCAPAKSE

FIG 14

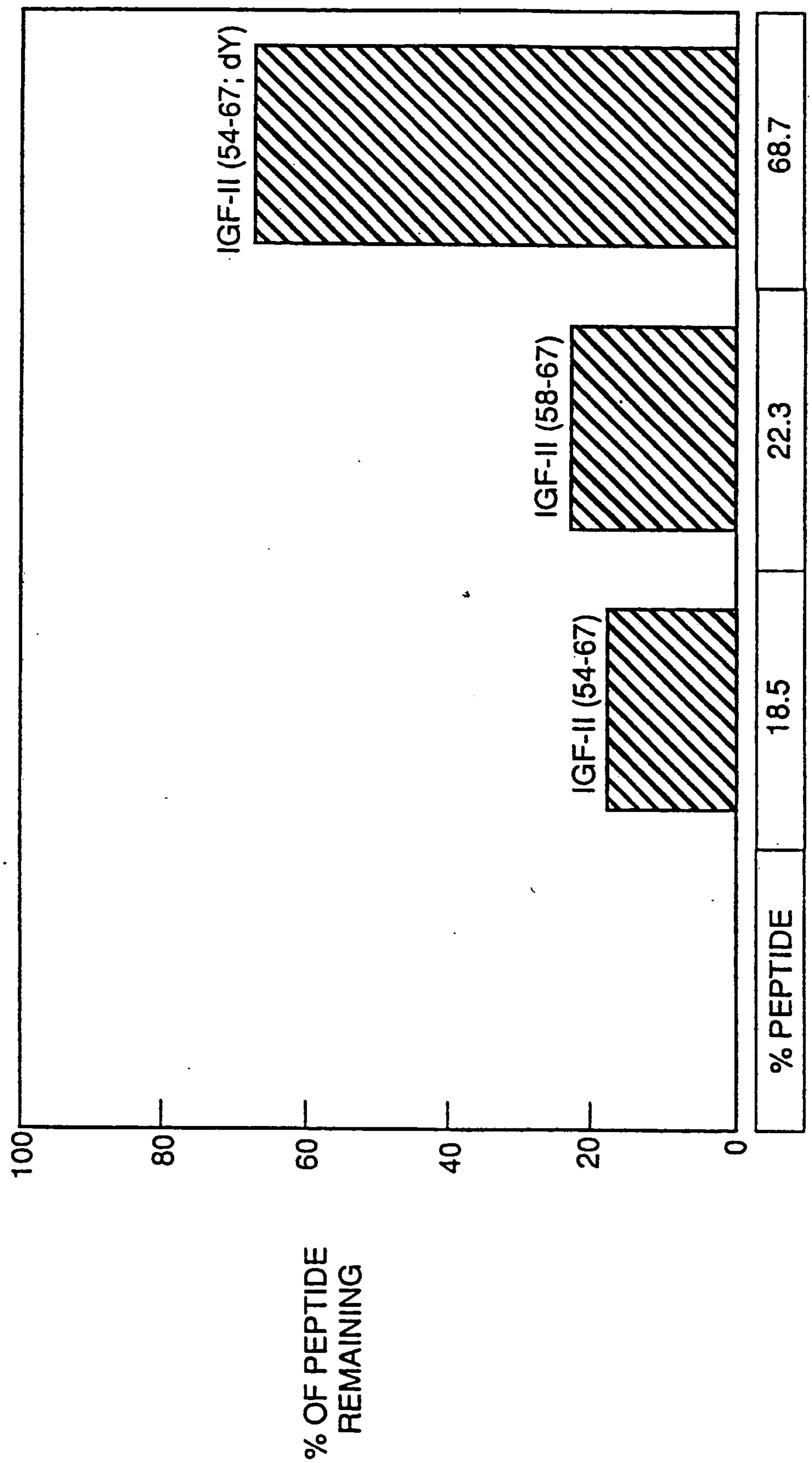

% OF PEPTIDE REMAINING
100
80
60
40
20
0
IGF-II (54-67)
IGF-II (58-67)
IGF-II (54-67; dY)
% PEPTIDE
18.5
22.3
68.7

FIG 15

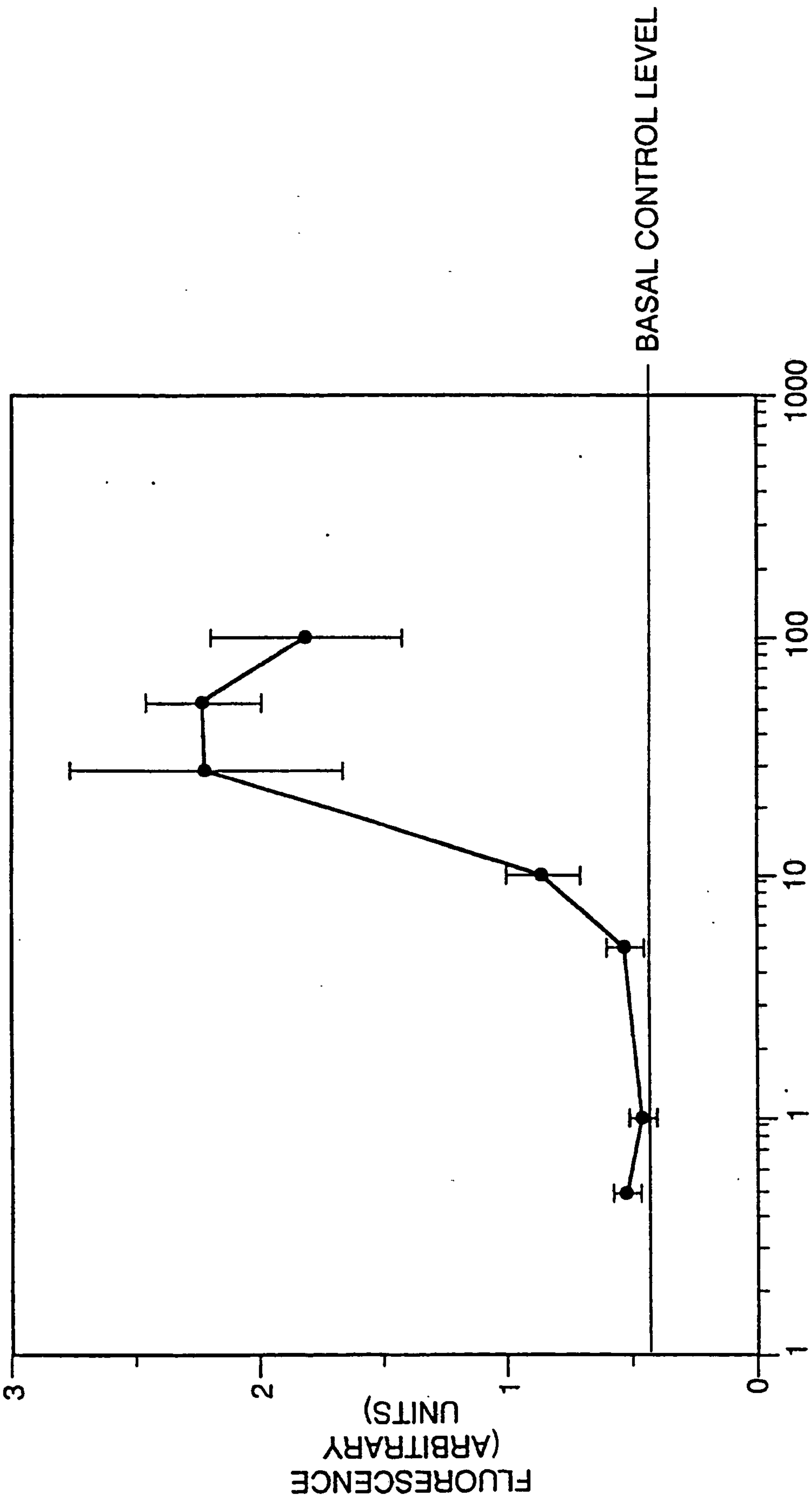

BASAL CONTROL LEVEL
FLUORESCENCE (ARBITRARY UNITS)
0
1
2
3
CONCENTRATION (uM)
1
10
100
1000

FIG 16

0 uM PEPTIDE
TYCAPAKSE

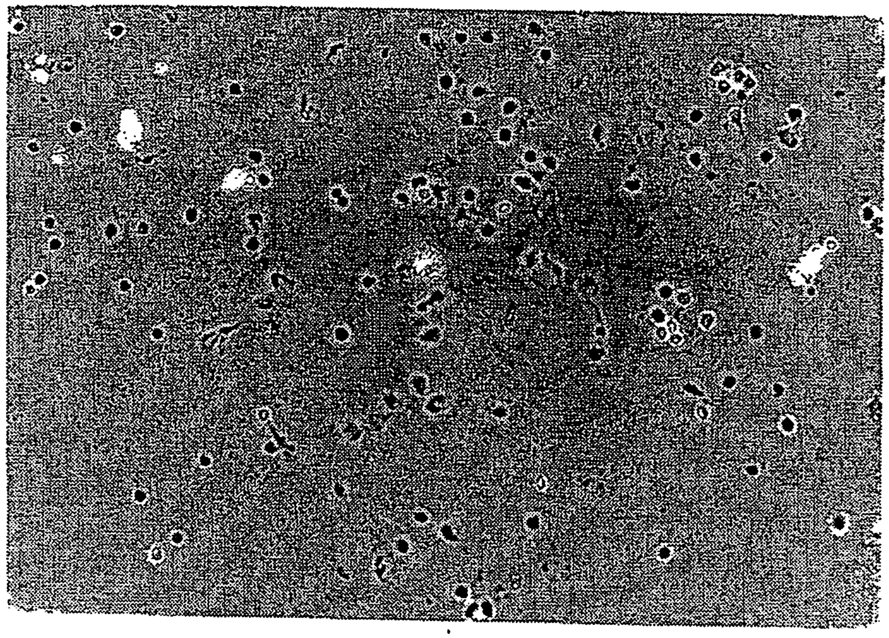


FIG. 17a

100 uM PEPTIDE
TYCAPAKSE

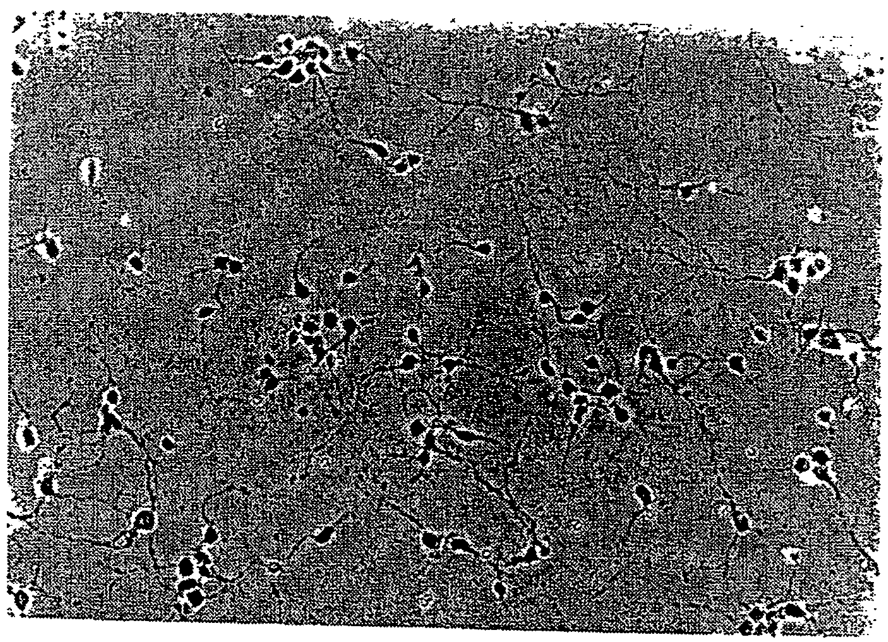


FIG. 17b

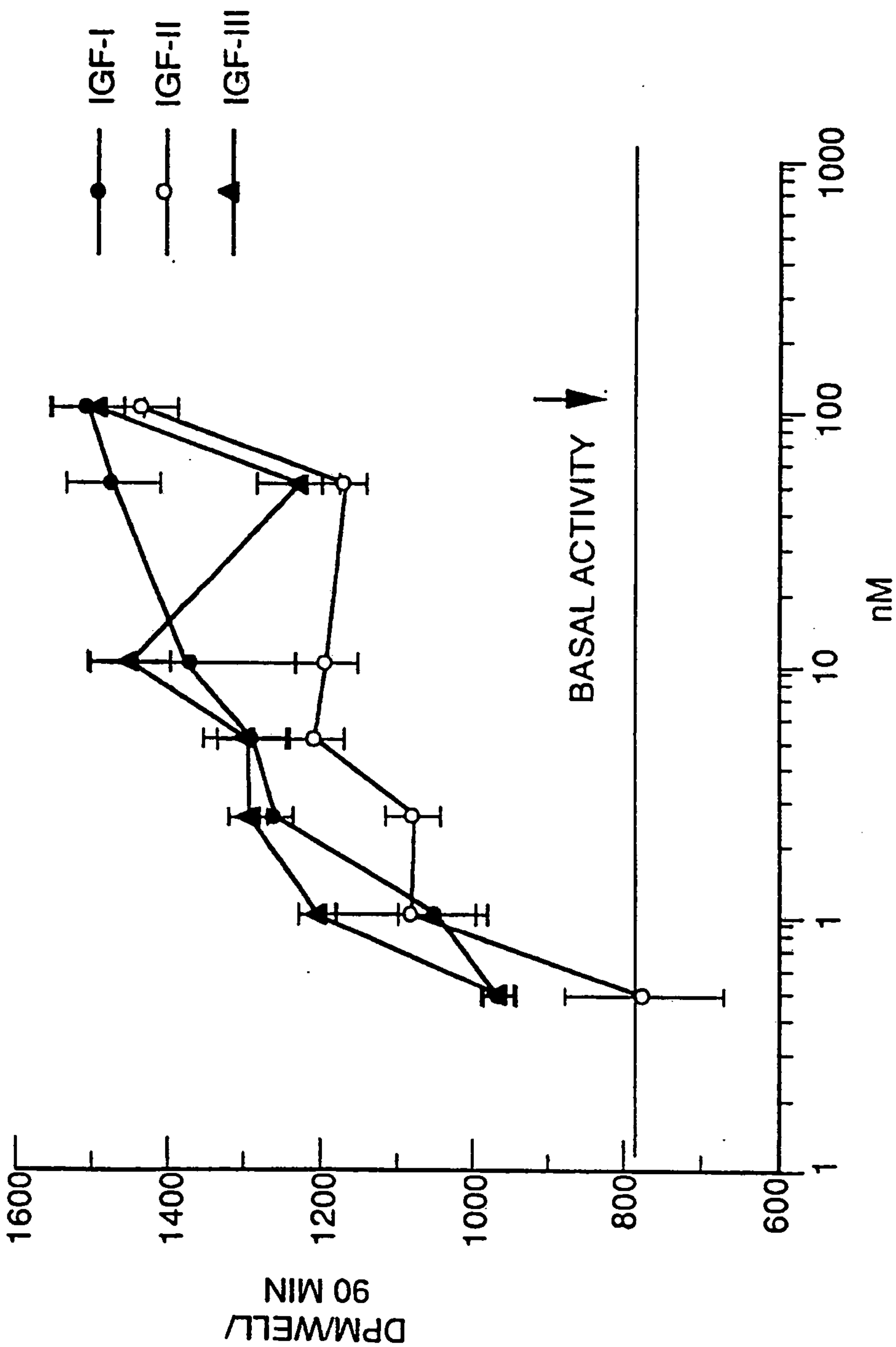

IGF-I
IGF-II
IGF-III
BASAL ACTIVITY
DPM/WELL/
90 MIN
1600
1400
1200
1000
800
600
1
1
10
100
1000
nM

FIG 18

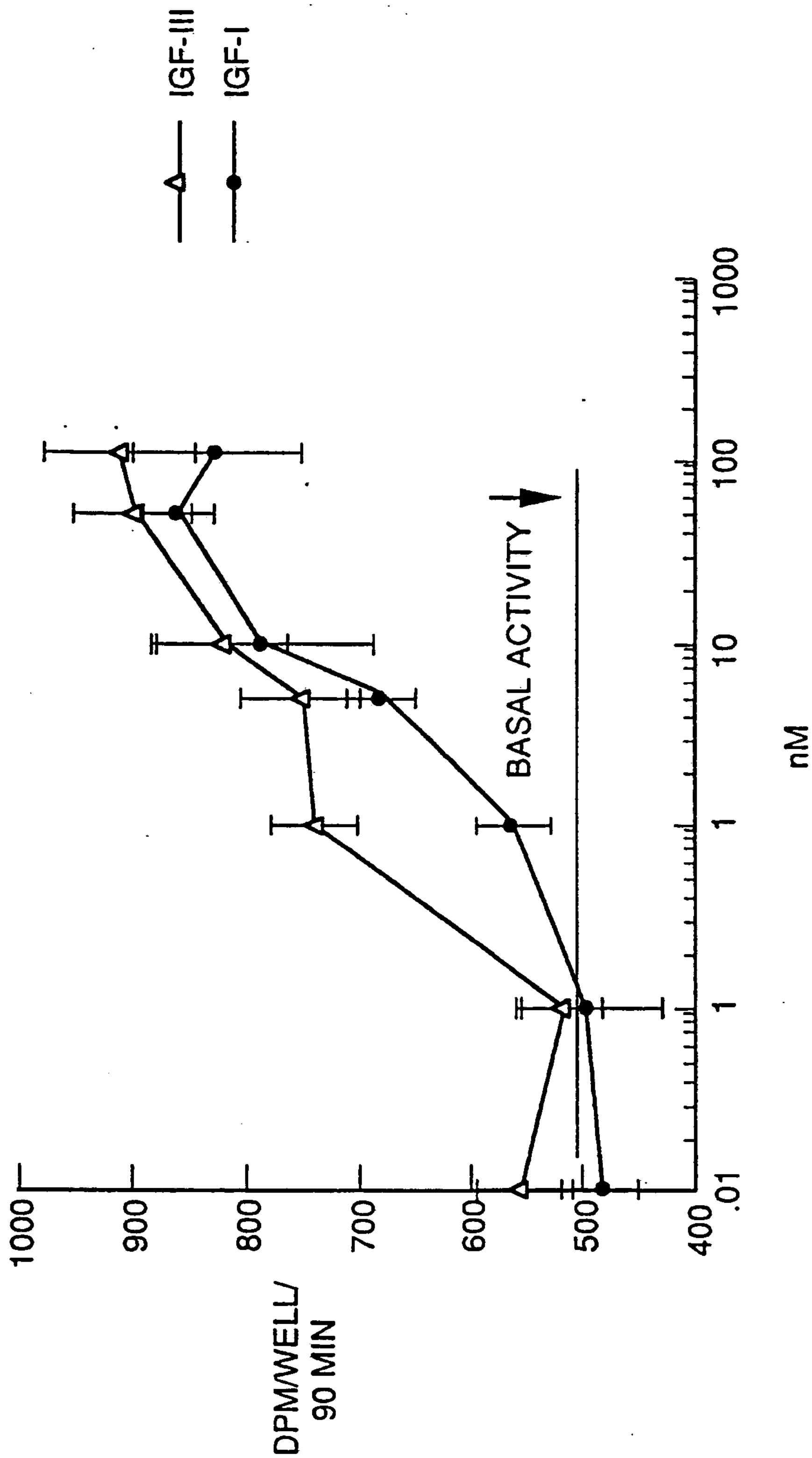

IGF-III
IGF-I
DPM/WELL/
90 MIN
1000
900
800
700
600
500
400
.01
.1
1
10
100
1000
nM
BASAL ACTIVITY

FIG 19

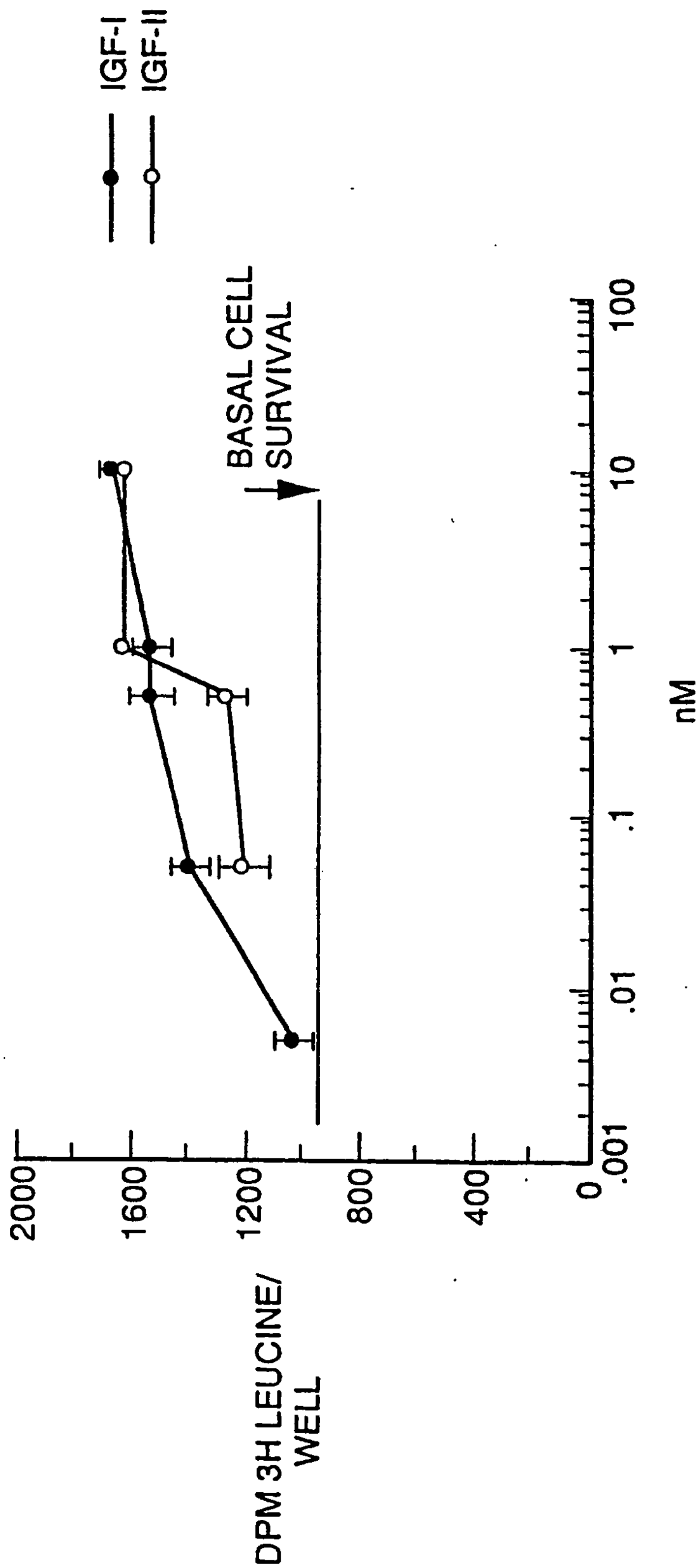

IGF-I
IGF-II
BASAL CELL SURVIVAL
DPM 3H LEUCINE/ WELL
2000
1600
1200
800
400
0
.001
.01
.1
1
10
100
nM

FIG 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03515

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02, 37/36; C07K 5/12, 7/06, 7/08, 7/10, 7/40.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/3, 4, 12, 13, 14, 15, 16, 17, 885, 903; 424/556, 570; 530/324, 325, 326, 327, 328, 329, 330, 412, 416.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS IGF, Age (W) related

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A 5,093,317 (Lewis et al.) 03 March 1992, see the entire document particularly the abstract.	1-36
A	US, A, 4,801,575 (Pardridge) 31 January 1989, see the abstract.	1-36
Y	Biochemical and Biophysical Research Communications, vol. 73, No. 3, issued 1976, D.M. Coy et al. "Synthesis and Opioid Activities of Sterioïsomers and Other D-Amino Acid Analogs of Methionine-Enkephalin" pages 632-638, see page 633, first paragraph.	2, 5, 6, 7, 8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 JULY 1993	21 JUL 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer Fatemeh T. Moezie
FATEMEH T. MOEZIE

Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Acta Physol. Scand., vol. 126, issued 1986, HA, Hansson et al., "Evidence Indicating Trophic Importance of IGF-I in Regenerating Peripheral Nerves", pages 609-614. See the entire document particularly the abstract.	7-10, 14, 35 36
Y	Neurobiology of Aging, vol. 3, issued 1982, V.R. Sara et al., "Somatomedins in Aging and Dementia Disorders of the Alzheimer Type", pages 117-120, see the entire document particularly the abstract.	1-36

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/3, 4, 12, 13, 14, 15, 16, 17, 885, 903; 424/556, 570; 530/324, 325, 326, 327, 328, 329, 330, 412, 416.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 289 314**
A2

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: 88303855.6

(22) Date of filing: 28.04.88

(51) Int. Cl.⁴: **A 61 K 37/36**
A 61 K 33/16

(30) Priority: 06.05.87 GB 8710676 28.04.87 US 43628

(43) Date of publication of application:
02.11.88 Bulletin 88/44

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

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Loma Linda, California 93257 (US)

(74) Representative: **Sheard, Andrew Gregory et al**
Kilburn & Strode 30, John Street
London WC1N 2DD (GB)

The title of the invention has been amended (Guidelines for Examination in the EPO, A-III, 7.3).
Claims for the following Contracting States: ES + GR.

(54) **Use of IGF-II in the treatment of bone disorders.**

(57) Peptides including Insulin-related Growth Factor-II (IGF-II) are useful in the treatment of bone disorders such as osteoporosis. They may be administered as the sole active ingredient of a pharmacological composition or in conjunction with a bone localising agent such as sodium fluoride, with which they have a synergistic effect.

EP 0 289 314 A2

Description

PHARMACOLOGICALLY ACTIVE PEPTIDES

This invention relates to the use of insulin-related growth factor-II (IGF-II) in pharmacological circumstances. It also relates to particular formulations of IGN-II.

IGF-II belongs to the family of growth factors that also comprises insulin, relaxin, insulin-like growth factor-I (IGF-I) and possibly the beta sub-unit of the 7s nerve growth factor - see Blundell and Humbel, Nature 287 781 (1980). IGF-II differs from the other members of its family by molecular weight amino acid sequence and the length of its connecting peptide. The primary structure of human IGF-II was first elucidated by Rinderknecht and Humbel, FEBS Letters 89 283 (1978). They give the amino acid sequence as:

AYRPSETLCGGELVDTLQFVCGDRGFYFSRPASRVSRRSRGIVEECCFRS CDLALLETYCATPAKSE

In the above structure, and throughout this specification, the single letter amino acid code can be correlated with corresponding three-letter notation as follows:

D=Asp E=Glu F=Phe K=Lys N=Asn
Q=Gln R=Arg W=Trp Y=Tyr M=Met
C=Cys L=Leu I=Ile H=His T=Thr
S=Ser P=Pro G=Gly V=Val A=Ala

The physiological role of IGF-II has been the subject of much speculation and little evidence. However, Brown et al, J. Receptor Res. 5 297 (1985) have shown that physiological concentrations of IGF-II stimulate thymidine incorporation by primary cultures of activated human T lymphocytes. IGF-II has been shown to enhance erythroid colony formation by human marrow cells (Dainniak and Kreczuko J. Clin. Invest. 76 1237 (1985)) and may be involved in the beta transforming growth factor-induced transformation of normal rat kidney (NRK) cells in soft agar (Massague et al, J. Biol. Chem. 260 455 (1985). Serum levels of IGF-II determined by radio immunoassays or radio receptor assays were not significantly different in acromegalics or growth hormone deficient subjects but are significantly elevated in the third trimester of pregnancy to return to lower levels after delivery (Wilson et al, J. Clin. Endo. Meta. 55 858 1982). There is a decline in serum IGF-II levels with age in normal women (644 ± 136 ng/ml in the eighth decade v 723 ± 217 ng/ml in the third decade, N = 57).

In spite of these observations, Zumstein et al (in PNAS 82 3169 (1985)) were moved to say that though IGF-I appeared to be a growth factor mediating the growth effects of and being under control of growth hormone, "the function of IGF-II is less clear".

Different animals may have different IGF-II molecules. In addition, Zumstein et al (Op. cit.) have identified and sequenced a variant IGF-II.

EP-A-0135094 discloses the amino acid sequence of IGF-I and IGF-II and nucleotide sequences of genes encoding them. Various hypotheses for their utility are put forward, but nothing concrete by way of clinically ueful activity is proposed, at least for IGF-II, other than the treatment of pituitary dwarfism.

WO-A-8600619 discloses prepro IGF-I and prepro IGF-II, but again gives no clinical utility for the end peptides.

EP-A-0193112 discloses cDNA encoding IGF-II. Again, no specific clinical utility is disclosed or forecast for IGF-II.

EP-A-0128733 discloses the production of "various forms" of human IGF and EGF (Epidermal Growth Factor) by recombinant DNA technology. There is no specific disclosure of clinical utility beyond saying that human IGF can be used as a human growth factor.

It has now been discovered that IGF-II can be of use in the treatment of bone disorders.

For several years it has been suspected that differentiation of bone forming tissues and the growth of differentiated bone is the result of the action of local tissue factors and that the coupling of bone formation to resorption provides a local mechanism for site-specific maintenance of endosteal bone volume. Illustrative publications on this subject include:

Farley et al, Program and Abstracts of 61st Annual Meeting of the Endocrine Society (1979);
Howard &; Baylink, Clinical Research 28 50A (1980);
Howard et al, Calcif. Tissue Int. 31 (Suppl.) 53 (1980);
Baylink &; Liu, J. Periodontol. 50 43-49 (1979);
Ivey &; Baylink (Editorial) Metab. Bone Dis. Relat. Res. 3 3-7 (1981);
Harris &; Haeny, New Engl. J. Med. 280 253-259 (1969);
Howard et al, Metab. Bone Dis. Relat. Res. 2 131-135 (1980);
Howard et al, PNAS USA 78 3204-3208 (1981);
Drivdhal et al, Proc. Soc. Exp. Biol. Med. 168 143-150 (1981) and
Drivdhal et al, Biochem. Biophys. Acta 714 26-33 (1982).

The possibility of there being a localised factor which controls bone growth offers the opportunity to isolate a material which could be effective in the stimulation and therapeutic control of bone growth in a wide variety of

situations. This is the role now proposed for IGF-II. IGF-II could therefore be useful in disorders of bones caused by disease. It may thus be used in methods of treating and preventing the bone wasting disorders known as osteopenias, particularly osteoporosis (whether idiopathic or secondary).

By osteopenia is meant disorders of bone such as osteogenesis imperfecta, osteomalacia, osteitis deformans, osteoporosis, rickets, fibrous dysplasia and the like. By osteoporosis is meant idiopathic wasting disorders of bone such as osteoporosis of aging, or osteogenesis imperfects, and secondary osteoporosis (e.g. resulting secondarily to other disease states such as eunuchoidism, hyperthyroidism, Cushing's syndrome, hyperparathyroidism, hypopituita-rism, gluten enteropathy, post-gastrectomy syndrome, glomeruloosteodystrophy, tubular disorders, vitamin D intoxication, immobilization, respiratory acidosis or inadequate dietary intake of vitamin C, calcium, phosphorous or protein). However, it is also envisioned that Insulin-like growth factor-II (IGF-II) could be useful in the treatment of impaired bone growth or in the enhancement of traumatic or surgical fracture repair, wherein surgical fractures include bone implants, or augmentation of bony incorporation of prostheses. Other sources of bone disease include infections (such as tuberculosis and syphilis) and neoplasms (whether bone forming tumours, chondroid tumours or other neoplasms such as Ewing's sarcoma or giant cell tumour).

It is therefore now envisaged that IGF-II could be useful in the treatment of (among other disorders of the bone) osteoporosis, in the treatment of impaired bone growth, in enhancement of fracture repair, possibly in conjunction with bone implants, and in the development and production of antibodies and reagents useful for diagnostic assays for the investigation of bone growth and bone degradation.

Osteoporosis is a bone wasting disease characterised by the loss of bone mass leading to atraumatic fracture. There is a resulting rarefaction of bone to leave the skeleton weakened and unable to bear the normal stresses placed upon it. The disease is particularly prevalent in the parts of the skeleton that are weight bearing, especially the spine, wrist and hips. The aetiology of the disease is multi-factorial. One especially common form of the disease occurs after the menopause. In the United States alone, post-menopausal osteoporosis effects 15 million women; it therefore poses a significant health problem.

Because the susceptibility to osteoporosis in a well-defined population, post menopausal women, is particularly high, and because of the osteoblastic enhancing activity of the pharmaceutical agents of the present invention, these agents may be utilized prophylactically to enhance or maintain osteoblastic (bone building) activity in the patient, thereby preventing the onset of the predominant osteoblastic (bone resorbing) activity observed in the disease state. However, forms of osteoporosis exist other than post menopausal, including primary and many forms of secondary osteoporosis. These agents may also be used therapeutically or prophylactically in the treatment of all forms of osteoporosis, whether primary or secondary, and in all bone wasting diseases.

According to a first aspect of the present invention, there is provided a peptide which is identical to or substantially homologous with an IGF-II, or an active subfragment thereof, for use in human or veterinary medicine.

One particular field of medicine is the treatment of bone disorders (whether resulting from bone disease, infection, neoplasms, surgery, fracture or otherwise). Therefore, according to a second aspect of the invention, there is provided the use of a peptide which is identical to or substantially homologous with an IGF-II, or an active subfragment thereof, in the preparation of an agent for use in bone disorders.

The IGF-II is preferably human IGF-II. However, it may be an IGF-II from another animal (particularly in veterinary applications). Therefore, the invention encompasses the use of bovine IGF-II and avian (for example chicken) IGF-II.

Although it may in some circumstances be preferred to use a peptide which is identical to natural IGF-II of a particular species, it may not always be essential. The use of homologues or variants is therefore contemplated, as is the use of active subfragments. Post-transcriptional or other modifications (such as glycosylation) are also contemplated. As stated above, the sequence of natural human IGF-II is:

AYRPSETLCGGELVDTLQFVCGDRGFYFSRPASRVSRRSRGIVEECCFRS CDLALETYCATPAKSE

and this sequence in some embodiments is preferred.

In the treatment of bone disorders, IGF-II may be administered parenterally. Therefore, according to a third aspect of the present invention, there is provided a sterile preparation of a peptide which is identical to or substantially homologous with an IGF-II, or an active subfragment thereof.

It has also been discovered that there may be advantages in adminstering IGF-II in combination with a bone localising agent. This may help avoid or at least mitigate any non-specific (extra skeletal) effects of IGF-II.

According to a fourth aspect of the present invention, therefore, there is provided a composition comprising a peptide which is identical to or substantially homologous with IGF-II, or an active subfragment thereof, and a bone localising agent. A preferred bone localising agent is a fluoride compound. Sodium fluoride is especially preferred. The bone localising agent helps target the IGF-II to bone sites.

According to a fifth aspect of the present invention there is provided a a pharmaceutical agent for treating an osteopenia in mammals comprising a therapeutically effective amount of Insulin-like Growth Factor II or a therapeutically effective amount of an osteoblastic stimulating fragment of Insulin-like Growth Factor-II, preferably in combination with a potentiating amount of fluoride ion, in a pharmaceutically acceptable carrier.

The pharmaceutical agent is advantageously either a parenteral unit dosage form, or in an aqueous

pharmaceutical carrier and suitable for intravenous injection, or in lyophilized form and upon rehydration is suitable for intramuscular administration.

A particularly preferred pharmaceutical agent is one in which the Insulin-like Growth Factor II has the amino acid sequence:

```
Ala-Tyr-Arg-Pro-Ser-Glu-Thr-Leu-Gly-Gly-
                                      10
Gly-Glu-Leu-Val-Asp-Thr-Leu-Gln-Phe-Val
                                      20
Cys-Gly-Asp-Arg-Gly-Phe-Tyr-Phe-Ser-Arg-
                                      30
Pro-Ala-Ser-Arg-Val-Ser-Arg-Arg-Ser-Arg
                                      40
Gly-Ile-Val-Glu-Glu-Cys-Cys-Phe-Arg-Ser-
                                      50
Cys-Asp-Leu-Ala-Leu-Leu-Glu-Thr-Tyr-Cys
                                      60
-Ala-Thr-Pro-Ala-Lys-Ser-Glu
```

or one substantially homologous therewith.

It appears that peptides usable in accordance with the invention may enhance bone cell division, bone matrix production and thereby eventually stimulate bone formation to increase the total bone volume of the body. In combination with a bone localising agent such as fluoride, the peptides may act synergistically to stimulate bone formation.

The present invention is at least to some extent based on the hitherto unpublished discovery that IGF-II is the same as skeletal growth factor (SGF). In particular, human IGF-II has been found to be identical to human SGF. SGF has been implicated in the stimulation of collagen synthesis (Linkhart et al, J. Cell. Physiol. 128 307 (1986)) and has been recognised as having a mitogenic effect on bone cells (Farley et al Biochemistry 21 (14) 3509 (1982)).

Although Canalis &; Raisz reported some years ago (Cacif. Tissue Int. 29 33-39 (1979) that Multiplication-Stimulating Factor (MSF) had the effect of stimulating DNA, collagen and non-collagen protein synthesis in bone cultures, this observation on its own is not regarded as significant in view of the large number of substances reported to have a similar effect.

IGF-II may be prepared by a variety of techniques. First, it may be extracted. Extraction may be from plasma as described by Rinderkencht and Humbel (Op. Cit.) Alternatively, it may be isolated from human bones, as described by Mojan et al Biochem. Biophys. Acta 884 234 (1986)). It should be noted that in the Mojan paper IGF-II was referred to as SGF. IGF-II may also be isolated from bone cells grown in culture.

Bovine and avian IGF-II may be similarly isolated from plasma or bone of the appropriate animal.

The second way in which the IGF-II may be repaired is peptide synthesis. As the natural human IGF-II is only 67 amino acids in length, it may be found to be fairly simple to prepare at least small quantities of the peptide by this technique.

The third (and what may ultimately be the preferred) method of preparation, however, involves recombinant DNA techniques. The first stage in such techniques would be to obtain a length of DNA coding for the desired IGF-II. One way to do this would be to isolate mRNA from IGF-II-producing cells and, with the in vitro use of reverse transcriptase, produce cDNA coding for the desired peptide. Alternatively, the DNA may be chemically synthesised. A number of oligonucleotides may be produced, from which the desired cDNA can be prepared by the use of DNA polymerase and DNA ligase. Restriction endonuclease digestion of either end can leave appropriate cohesive restrictions sites for insertion into a plasmid.

Whether the synthetic DNA is cDNA or chemically synthesised, it can either have cohesive ends provided by a restriction endonuclease or it may be terminally tailed by for example oligo-dC by the use of the appropriate nucleotide and terminal transferase.

Whichever tailing method is chosen, a plasmid (for example pBR322) can then be taken and cleaved at a single site by a restriction endonuclease such as PstI. PstI cleaves pBR322 in the gene coding for ampicillin resistance. This allows for easy selection of recombinant plasmids. If desired, the PstI-digested pBR322 can be oligo-dG tailed to complement an oligo-dC tail piece of DNA coding for the desired peptide. The cleaved

plasmid and the DNA coding for the peptide can be annealed and ligated; host cells (for example E. coli) can be transformed with the appropriate recombinant plasmid.

The transformed E. coli host cells may be cultured under appropriate conditions to express the IGF-II peptide.

It should be noted that it is not envisaged that production by recombinant DNA techniques should be limited to bacterial systems. Eukaryotic systems such as yeast systems can also be used and may in practice be preferred.

The invention is now illustrated by the following examples.

EXAMPLE 1

Production of IGF-II by bone cells.

Human bone cells are isolated from femoral heads obtained during hip replacement surgery. They are grown in monolayer culture in dishes containing Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. The release of IGF-II into the culture medium can be followed using a radioreceptor assay, using rat hepatoma (H35) cells in monolayer culture and IGF-II release is seen to increase with increasing culture time. The results are shown in Table 1.

The production of IGF-II is modulated by systemic agents. In the human bone cell monolayer culture model described above, the addition of insulin (10 to 10,000 ng/ml) for 24 hours stimulates the production of IGF-II by the bone cells in a dose dependent manner. This again can be seen from Table 1.

TABLE 1

Effect of insulin on the secretion of IGF-II by human bone cells *in vitro*.

Insulin (ng)	IGF-II levels (ng/ml) after insulin exposure of 0-24 hours	24-48 hours
0	17.2 + 5.0	12.5 + 5.0
10	16.7 + 1.0	12.0 + 5.0
100	22.6 + 8.5	16.0 + 9.0
1000	24.6 + 6.5	19.0 + 11.5
10000	32.0 + 7.0	28.0 + 1

Treatment of cultured human bone cells with human growth hormone (10 ng/ml) for 5 days increases IGF-II production by 50% (p <; .01). Additionally, human somatostatin (50 ng/ml) also stimulates IGF-II production by human bone cells in culture by 148% compared with untreated controls (p <; .01).

Human bone cells produce very much more IGF-II than IGF-I. Media conditioned by 2 × 10^4 cells for 24 hours contain 17.6 ng/ml of IGF-II. During the same interval under identical culture conditions, the cells produced only 1.16 ng/ml of IGF-I. Thus, human bone cells are shown to produce more than 15 times more IGF-II than IGF-I.

EXAMPLE 2

Extraction of IGF-II from bone matrix.

Human femoral heads were obtained during hip replacement surgery at local hospitals. They were cleaned with a knife to remove the adhering soft tissue. The bones were then cut into small pieces (approximately 2 cm^3) using a band saw and washed with cold tap water to remove the blood and marrow. The bone pieces were frozen in liquid nitrogen and ground with solid CO_2 using a Wiley mill. the resulting bone powder (2 mm^3) was washed overnight by constant mixing with deionized water containing proteinase inhibitors (5 mM benzamidine, 100 mM epsilon-aminocaproic acid and 1 mM phenylmethylsulphonyl fluoride). After washing with deionized water, the residue was extracted for 72 to 96 hours by constant stirring with excess (5 vol.) 30 mM tris-acetate/4 M guanidine-HCl (pH 7.4) containing proteinase inhibitors. By this extraction, serum protein (albumin, immunoglobulins etc) and other loosely bound proteins were extracted. The supernatant was decanted and the residue was re-extracted for an additional 24 hours with 4 M guanidine solution. After the guanidine extraction, the residue was demineralised for 7 days by constant mixing with 10% EDTA/4 M guanidine-HCl containing proteinase inhibitors (pH 7.4). The supernatant was decanted, centrifuged (10,000 rpm for 20 minutes), filtered and concentrated by Amicon membrane filtration. The concentrate (guanidine-EDTA extract) was washed with excess of 4 M guanidine-HCl to remove EDTA completely. The guanidine EDTA extract was used for further human IGF-II purification under dissociative conditions. The guanidine EDTA extract was dialysed against distilled water using Spectrapor membrane tubing (molecular weight cut-off 3500), assayed for protein concentration and tested for mitogenic activity.

IGF-II activity in the guanidine-EDTA extract was purified by hydroxyapatite and gel filtration chromatography as follows.

Hydroxyapatite (Fast Flow, Bio-Rad) was equilibrated with 30 mM tris-acetate/4 M guanidine-HCl/10 mM potassium phosphate (pH 7.4). The phosphate concentration of the guanidine-EDTA extract was adjusted to 10 mM and the sample was applied to the hydroxyapatite column (15 × 5 cm). The unbound proteins were eluted with 10 mM phosphate in 30 mM tris-acetate/4 M guanidine-HCl (pH 7.4). The bound proteins were eluted in a single step by increasing the phosphate concentration to 400 mM in the same buffer. Aliquots of unbound and 400 mM phosphate-eluted fractions were desalted by dialysis and tested for protein concentration and mitogenic activity.

The unbound fraction from hydroxyapatite chromatography (which contained 90% of the mitogenic activity) was concentrated and the proteins were separated by HPLC gel-filtration chromatography on a preparative TSK- G3000 SWG column (21.5 × 600 mm, LKB products). The chromatography was performed with a Beckman Model 344 gradient liquid chromatography system, which consists of two Model 112 pumps controlled by a Model 421 controller-programmer. Two ml samples were applied to the column with an Altex Model 210 injection valve and the proteins were eluted at 2 ml/min with 30 mM trisacetate/4 M guanidine-HCl (pH 7.4). The absorbence was monitored at 280 nm (Beckman Model 160 detector) and the elution profile was recorded using a Hewlett Packard Integrator Model 3390A. 2-min fractions were collected and the fractions were pulled according to the protein peaks and concentrated. Aliquots of the pools were dialysed against distilled water to remove guanidine and assayed for protein content and mitogenic activity.

The active pool obtained from gel filtration (6 to 17.5 kDa) was dialysed against 10 mM tris-HCl, pH 7.2 containing 100 mM NaCl and was loaded in 10 ml of the same buffer into a 0.5 × 10 cm heparin-sepharose affinity column (Pharmcia FPLC System). The unbound proteins were eluted with the starting buffer (20 minutes, Flow Rate 1 ml/min, 2 minute fractions) and the bound proteins were eluted by a gradient of 0.1 to 3.0 M sodium chloride over 10 minutes. Fractions were diluted in 1 mg bovine serum albumin per ml DMEM and tested for biological activity using [^{3}H] thymidine incorporation into the DNA of chick embryo bone cells in serum-free monolayer culture as described in Linkhart et al Bone 7 479-487 (1986).

The active fractions from heparin-sepharose chromatography (fractions 15 to 30, 0.2 to 0.6 M NaCl) were pooled and re-run on the same heparin-sepharose column using the same gradient. The active fractions from the second heparin-sepharose step (fraction 17 to 22) were pooled, concentrated and then subjected to two sequential separations by reverse phase chromatography in 0.1% trifluoroacetic acid (TFA) using a 4.6 × 250 mm C_4 column (Bio-Rad RP 304). The first separation used a 10 to 60% acetonitrile gradient in 50 minutes and the second separation used a 25 to 45% acetonitrile gradient in 100 minutes. Final purification of human IGF-II was achieved by HPLC reverse phase chromatography in 0.1% TFA using a 3.9 × 150 mm Microbondapak CN column (Waters Corporation) with a 1-propanol gradient (20 to 40% in 100 minutes). HPLC reverse phase chromatography was done using a Beckman Model 344 gradient liquid chromatography system with a 214 NM detector. Fractions were concentrated by Speed-Vac Evaporation (Savant Instruments).

EXAMPLE 3

IGF-II is active on bone cells.

IGF-II, as obtained in Example 1 or 2, stimulates division in bone cells in monolayer culture and is therefore a bone cell mitogen. This is demonstrated using embryonic chick calvarial cells in monolayer culture. The results are shown in Table 2.

TABLE 2A

Data are mean ± standard deviation of six replicates (basal counts = 351 ± 46, n=6).

IGF-II (ng/ml)	3H-TDR INCORPORATION (% of unstimulated control)
0	100 ± 13
0.3	202 ± 32
1.0	269 ± 46
3.0	338 ± 39
10.0	403 ± 54
30.0	412 ± 20

In a similar experiment conducted on human bone cells, purified human IGF-II at a concentration of 2ng/ml stimulated the proliferation of human bone cells at 362% of the Control value (Table 2B).

Table 2B Effect of Human IGF-II on the Proliferation of Human Bone Cells.

Treatment	Concentration	CPM	% of Control	Significance
Control		316 ± 81	100	
human IGF-II[a]	2 ug/ml	1146 ± 80	362 ± 80	p <.001
serum	1%	2479 ± 816	897 ± 165	p <.001

Data are expressed as mean + 1 SD of six samples, Significance expressed compared to control (Students t Test). a = Partially purified human IGF-II

This enhanced mitogenic activity in the chick and human bone cell cultures, which was stimulated by IGF-II, is directly translatable into enhanced osteoblastic (bone producing) activity within the bone. It is to be noted that when purified human IGF-II was added to cultures of embryonic mouse calvariae pre-labelled with the isotope ^{45}calcium, IGF-II had no significant effect on the release of the ^{45}calcium from these bones, indicating

a lack of any stimulatory affect on bone resorption.

Thus, by using IGF-II in therapeutically effective amount, it is possible to increase the osteoblastic activity within the bone to a point exceeding the pre-existing osteoclastic activity so as to effect a cure in a patient suffering from an osteopenia.

Similarly, IGF-II can be administered to a patient susceptible to an osteopenia in a prophylactically effective amount, so as to maintain a balance between osteoblastic and osteoclastic activity in said patient.

In a particularly preferred embodiment of the present invention, the osteopenia being treated or prevented is osteoporosis.

EXAMPLE 4

IGF-II when combined with fluoride is targetted to bone.

When labelled with fluoride, IGF-II is bone-specific in its actions. Additionally, when combined with sodium fluoride, IGF-II acts synergistically with this drug to stimulate bone cell division and bone matrix production above the levels achieved by either agent when used alone. Specifically thymidine incorporation into DNA is increased, proline incorporation into collagen is stimulated and cellular alkyline phosphatase is increased by the drug combination.

Thus while both IGF-II and fluoride ion increase the proliferation of human bone cells (Table 2C) as determined by the incorporation of tritiated thymidine into DNA, the combination of IGF-II and fluoride ion produces an unexpectedly enhanced effect upon bone cell proliferation, said fluoride ion potentiating the osteoblastic stimulating activity of IGF-II (Table 2C). In addition, fluoride is the single most effective agent for restoring bone mass lost as a result of osteoporosis, and, at levels that are effective in stimulating bone formation, fluoride particularly as a slow release preparation does not have appreciable side effects on other organ systems.

Table 2C Effect of IGF-II and Fluoride on Thymidine Incorporation into DNA by Cultured Human Bone Cells and Human Skin Fibroblasts.

CELL TYPE	PERCENTAGE OF CONTROL BOVINE IGF-II (8.5 UG/ML)	FLUORIDE (20mM)
Human bone	370 ± 62 p .01	252 ± 52 p <.01
Chick bone	538 ± 71 p .01	140 ± 38 p <.05
Human skin	271 ± 84 p .01	87 ± 3

Data expressed as mean ± SD, Control incorporation rates in cpm were 430 ± 400 (Human bone), 315 ± 50 (chick bone), and 1019 ± 485 (human skin).

Accordingly, the combination of IGF-II and fluoride ion, wherein the fluoride ion (in a physiologically acceptable form such as MFP, NaF or other) is associated with a pharmaceutically acceptable cation, would provide an especially effective pharmaceutical agent in the treatment of the osteopenias, particularly osteoporosis.

Such pharmaceutically acceptable cations include sodium, potassium, lithium, calcium, magnesium, ferrous, zinc, copper, manganous, aluminum, ferric, manganic and the like. Particularly preferred are the ammonium, potassium, sodium, calcium and magnesium salts.

It is well known in the art that fragments of biologically active polypeptides, either alone or attached to other non-interferring peptides, polysaccharides, aldoses, and like molecules, can possess substantially the same biological activity as the parent intact polypeptide. Accordingly, this invention also encompasses fragments of IGF-II also possessing osteoblastic stimulating activity, whether obtained from an intact molecule or

synthetically such as by chemical manipulation or by recombinant DNA techniques.

It is also within the scope of this invention to employ said osteoblastic stimulating fragments alone or in combination with fluoride ion to treat or prevent the osteopenias, particularly osteoporosis, in patients suffering from or susceptible respectively to said diseases states.

A veterinarian or physician of ordinary skill can readily determine whether a subject exhibits a bone wasting condition, osteopenia.

The compounds of this invention may be administered parenterally (other than by mouth) such as intravascularly, intraperitoneally, subcutaneously, intramuscularly, or by suppository using forms known to the pharmaceutical art, or by transdermal route (air gun or skin patch delivery). In the case of fractures, the compounds may, in addition to the above methods, be administered locally, into, at or near the fracture site.

For intravascular, intraperitoneal, subcutaneous, or intermuscular administration, the active drug components of the present invention in liquid, powdered, or lyophilized form may be combined with a suitable diluent or carrier (collectively referred to herein as "carrier" materials) such as water, saline, aqueous dextrose, aqueous buffers, and the like. Preservatives may also be added.

Regardless of the route of administration selected, the compounds of the present invention are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those skilled in the art. The compounds may also be formulated using pharmacologically acceptable acid or base addition salts. Moreover, the compounds or their salts may be used in a suitable hydrated form.

Regardless of the route of administration selected, a non-toxic but therapeutically effective quantity of one or more compounds of this invention is employed in any treatment. The dosage regimen for preventing or treating a bone wasting condition with the compounds of this invention is selected in accordance with a variety of factors, including the type, age, weight, sex, and medical condition of the patient, the severity of the inflammatory condition, the route of administration, and the particular compound employed in the treatment. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent or arrest the progress of the condition. In so proceeding, the physician or veterinarian could employ relatively low doses at first and subsequently increase the dose until a maximum response is obtained.

EXAMPLE 5

Antibody-mediated extraction of IGF-II from bone matrix.

IGF-II antibodies (monoclonal or polyclonal) may be used to extract IGF-II from bone matrix using an affinity column. Alternatively, IGF-I may be used. The reasons that IGF-I antibodies work are because (a) IGF-II is present in a relatively high concentration in bone compared to IGF-I and (b) IGF-II binds to IGF-I antibodies.

EXAMPLE 6

Purification of IGF-II from serum.

Using conventional techniques, bone cell derived IGF-II may also be purified from animal serum. Mammalian serum, including human, bovine, ovine, porcine or equine serum, may be used.

EXAMPLE 7

Preparation of Bovine Insulin-like Growth Factor II (bIGF-II) from Extract.

The hip bones from freshly slaughtered cows are mechanically scraped clean of soft tissue and cut into $2cm^3$ sections. The bone sections are frozen in liquid nitrogen and ground in a Wiley mill to yield a bone powder that is washed with warm water to remove fat and serum protein, demineralized and extracted using 20% EDTA, 0.04% sodium azide as described above. The EDTA extracts are concentrated and partially desalted by Amicon ultrafiltration. The remaining EDTA is removed by desalting with Sephadex G-25. Three alternative methods can be used for further purification of the desalted crude extracts.

1. Gel filtration on Agarose yields an active bIGF-II fraction having a MW range of 150 to 200kdal, probably consisting of bIGF-II bound to its binding protein or pre-IGF-II.

2. Direct purification of the crude extract on DEAE Sephacel yields a large bIGF-II fraction that is modestly bound. In addition, active bIGF-II fractions that are unbound or weakly bound to DEAE Sephacel are obtained in the range of 10 to 20kdal.

3. Purification of desalted crude extract on hydroxyapatite yields a non-active fraction that elutes with 0.15M phosphate and an active fractions that is recovered by dissolving the hydroxyapatite support with EDTA. The tightly bound fraction yields approximately equal amounts of large bIGF-II and small bIGF-II.

The large partially purified bIGF-II tends to become small on further manipulation. Evidence suggests that an endogenous bone protease is present in the bone extracts and is activated during the purification process. This protease is evident in the bovine bone extracts as will be indicated subsequently in chicken extracts, but is not observed in the purification of the human extracts.

EXAMPLE 8

Preparation of Chicken Insulin-like Growth Factor (II) (cIGF-II) from Extract.

The tibia and femur of adult chickens are mechanically scraped clean of soft tissue. The cartilage ends are cut off and the bones are frozen in dry ice and smashed into 3mm^3 pieces. Serum proteins and fat are removed by extensive washing of the bone pieces in 0.03M Tris(acetate), 0.15M NaCl, (pH 7.4), with vigorous agitation. The bone is then subjected to demineralization and extraction with 10% EDTA, 0.04% sodium azide. The EDTA extracts are concentrated and partially desalted by Amicon ultrafiltration and the remaining EDTA removed by Sephadex G-25 chromatography. Two approaches are used to further purify the cIGF-II.

1. Gel filtration of the desalted crude extract on Agarose 0.05M yields predominately a large form of cIGF-II (100 to 200kdal), as well as small quantities of the small cIGF-II in the 10 to 20kdal range.

2. DE-52 chromatography of the desalted crude bone extract yields a cIGF-II fraction that is weakly bound to the matrix. The weakly bound fraction behaves like large cIGF-II when chromatographed on a TSK 3000 HPLC gel filtration column shortly after isolation. Preincubation of the weakly bound fraction before HPLC gel filtration results in a shift of large cIGF-II to the smaller cIGF-II. This change in apparent size is believed to be a result of the presence of a protease which is detected in the fraction weakly bound to DE-52. The cIGF-II activity in the desalted crude extracts binds to hydroxyapatite, QAE cellulose and DE-52, but does not bind to collagen linked to Agarose or to carboxymethyl Sepharose.

EXAMPLE 9

Production of Human Insulin-like Growth Factor - II by Bone Cells in Culture.

Human bone cells were collagenase dissociated and grown in monolayer culture in 24 well dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HIGF-II is released into the culture medium by the bone cells as they grow.

As noted in the detailed discussion (Table 1), insulin can be added in varying amounts to increase the amount of hIGF-II produced. Similarly, human growth hormone (10 ng/ml) or somatostatin (50 ng/hl) can also be used to enhance hIGF-II production (see detailed discussion).

Extraction and purification are performed as in Example 1.

Two separate in vivo experiments were conducted on rats. In both experiments the rats in the Treated group were parenterally administered a bovine bone IGF-II extract. The rats in the Control group were administered a placebo. The data from Experiment 1 is presented in Tables 3 and 4; that of Experiment 2 in Tables 5 to 7.

Experiment 1

Experiment 1 consisted of 5 rats in the Control group and 3 rats in the Treated group.

In Table 3, the parameters measured in the Treated and Control groups consisted of the following (1) serum calcium; (2) body weight at the start of the experiment; (3) body weight at the end of the experiment; (4) femur alkaline phosphatase activity; and (5) femur acid phosphatase activity. As to the first three parameters, no significant (N.S.) difference was demonstrated between the Control group, which was not receiving IGF-II, and the Treated group, which was receiving bovine bone IGF-II extract.

However, as to parameter 4, the femur alkaline phosphates, there was a significant difference between the Treated and Control groups. Specifically, in the Treated Group, the femur alkaline phosphatase was increased 136% over that in the Control group. Increased alkaline phosphatase activity in bone by itself is suggestive of increased osteoblastic (bone building) activity. However, when combined with the data in Table 4, showing the results of histomorphometric assessment of the rat bones, the result is indicative of increased osteoblastic activity within the bone.

Table 3 In Vivo Effect of Bovine Bone IGF-II Extract on Rats (Experiment 1)

	Parameter	Control	Treated[a]	p[b]
1.	Serum Calcium (mg%)	9.8(±0.7)	9.0(±0.5)	NC[c]
2.	Body Weight (Start)	104(±3.7)	105(±4.2)	NS
3.	Body Weight (End)	147(±0.9)	147(±9.2)	NS
4.	Femur Alkaline Phosphatase (mU/mg protein)	4.9(±0.9)	11.6(±1.2)	<0.001
5.	Femur Acid Phosphatase Tartrate Insensitive (mU/mg protein)	3.4(±0.4)	4.8(±0.3)	<0.02

a All data reported as mean ±S.E.M. and based on observations from 5 rats in the control group and 3 rats in the treated group.

b Comparisons of treated versus control were by the students' T test.

c Not significant at the $p < 0.05$ student T test.

Table 4 Histological Assessment of In Vivo effect of Bovine Bone IGF-II Extract on Rats (Experiment 1)

	Parameter	Control	Treated[a]	p[b]
1.	Tibiae Periosteal Bone Formation (mm^3)	0.23($\pm$0.02)	0.40($\pm$0.02)	<0.001
2.	Tibiae Periosteal Bone	6.75($\pm$0.43)	8.60($\pm$0.46)	<0.05
3.	Tibiae Medullary Cavity (mm^3)	0.67($\pm$0.03)	0.81($\pm$0.09)	<0.05
4.	Vertebral Forming Surface (single + double label) % total surface	58.3($\pm$2.5)	90.5($\pm$1.7)	<0.001
5.	Vertebral Neutral Surface (no label) % total surface - V.F.S.)	41.7($\pm$1.9)	9.5($\pm$1.4)	<0.001

a All data reported as mean $\pm$S.E.M. and based on observations from 5 rats in the control group and 3 rats in the treated group.

b Comparisons of treated versus control were by the students' T test.

c Not significant at the p <0.05 student T test.

The most dramatic effect of IGF-II extracts on bone is seen in the data in Table 4. Table 4 reflects histological comparisons between the tibiae and vertebrae (bones) of the Treated and Control groups of rats from Experiment 1 after sacrifice. The (IGF-II extract) Treated group demonstrates markedly increased bone growth over the Control group in parameters associated with bone growth. Specifically, relative to the Control group, the Treated group demonstrated 73.9% more tibiae periosteal bone formation (the volume of new bone formed in the outer connective tissue, periosteum, covering the bone); and a 27.4% greater periosteal apposition rate (the rate of laying down of new bone) -- parameters 1 and 2 of Table 4.

Moreover, the IGF-II extract Treated group also exhibited a 55% increase in bone forming surface over the untreated Control group, as measured by the ability of the bone forming surface to pick up a label -- parameter 4 of Table 4. Alternatively, when this same data is viewed from the perspective of inactive bone forming surface, the data discloses that the Control group had 339% more inactive bone forming surface than the Treated group -- parameter 5 of Table 4.

Thus Experiment 1 demonstrates that parenteral administration of IGF-II extract stimulates significant osteoblastic activity in vivo.

The data in Tables 3 and 4, also suggest that additional activity within the bone may also be stimulated. The increase in tartrate insensitive acid phosphatase activity that was observed in the Treated group is likely due to increased osteoclastic (bone resorbing) activity. However, this was not verified by histological staining. The increase in the tibiae medullary cavities by 21%, parameter 3 of Table 4, is indicative of some increased osteoclastic (bone resorbing) activity.

This increase in osteoclastic activity, however, is due to the presence of small amounts of transforming growth factor (TGF) in the bovine bone extract. Later studies employing pure IGF-II to treat bone cells in culture demonstrate no such osteoclastic effect. Moreover, TGF is recognized as a potent stimulator of osteoclastic (bone resorbing) activity.

Experiment 2

A second more detailed experiment on the in vivo effects of parenteral administration of bovine bone IGF-II extract similarly indicates that IGF-II significantly increases osteoblastic activity within the bone (Tables 5-7).

In this second experiment, Experiment 2, ten rats were used in both the Treated and Control groups. As in Experiment 1, the serum calcium and body weight remained unchanged with treatment (Table 5).

In Experiment 2, the levels of bone alkaline phosphatase were analyzed more extensively. Again, the Treated group exhibited significantly increased levels of alkaline phosphatase in the femur, skull and sternum over that exhibited by the Control group, 83%, 165%, and 102%, respectively -- parameters 1, 2, and 3 of Table 6. The acid phosphatase levels, although increased, were only significantly increased ($p <;0.001$) in the femur and skull of the Treated group and by 93%, and 71% respectively.

The results of the alkaline phosphatase analysis in the femur, skull and sternum are consistent with an increase in osteoblast number and bone formation. The alkaline phosphatase data also agrees very well with the quantitative histology of the tibiae of treated rats, where a 60% increase ($p <;0.001$) in the periosteal bone formation (mm^3) and a 40% increase in the matrix apposition rate (/day) were observed. (Table 7).

As in Experiment 1, the increase in tartrate sensitive acid phosphatase in the femur and skull is consistent with an increase in the amount of osteoblastic acid phosphatase due to the increased number of osteoblasts. It is also consistent with the presence of the osteoclast stimulating agent, beta transforming growth factor (TGF), in the bone extract.

Table 5 *In Vivo* Effect of Bovine Bone IGF-II Extract on Rats (Experiment 2)

	Parameter	Control[a]	Treated[a]	p[b]
1.	Serum Calcium (mg%)	10.7(±0.3)	11.1(±0.3)	NS[c]
2.	Serum Phosphate (mg/dl)	4.7(±0.5)	5.8(±0.2)	<0.05
3.	Serum Alkaline Phosphatase (mU/mg protein)	63.7(±2.2)	91.7(±5.2)	<0.001
4.	Body weight (Start)	86.4(±0.2)	85.0(±0.6)	NS
5.	Body Weight	149.8(±2.6)	147.7(±2.5)	Ns

a All data reported as mean ±S.E.M. and based on observations from at least 10 rats in each group.

b Comparisons of treated versus control were by the students' T test.

c Not significant at $p < 0.05$.

Table 6 Effect of Bovine bone IGF-II Extract on Enzyme Activity within the Bones of Rats. (Experiment 2)

	Alkaline Phosphatase[a] (mU/gm bone wet weight)			Acid Phosphatase[a] Tartrate Insensitive (mU/gm bone wet weight)		
	Control	Treated	p	Control	Treated	p
Femur	155(±24)	284(±19)	<0.001	31(±5)	60(±7)	<0.001
Skull	405(±44)	1075(±84)	<0.001	355(±42)	607(±52)	<0.001
Sternum	46(±2)	93(±7)	<0.001	107(±16)	147(±14)	NS

a All data reported as mean ±S.E.M. and based on observations from at least 10 rats in each group.

b Comparisons of treated versus control were by the students' T test.

c Not significant at p <0.005.

Table 7 In Vivo Effect of Bovine Bone IGF-II Extract on the Tibiae of Rats (Experiment 2)

	Parameter	Control	Treated[a]	p[b]
1.	Tibiae Periosteal Bone Formation (mm^2)	0.45($\pm$0.02)	0.72($\pm$0.04)	<0.001
2	Tibiae Periosteal Bone Apposition Rate (/day)	8.58($\pm$0.33)	12.07($\pm$0.51)	<0.001

a All data reported at mean $\pm$S.E.M. and based on observations from at least 10 rats in each group.

b Comparisons of treated versus control were by the students' T test.

Claims

1. A peptide which is identical to or substantially homologous with an IGF-II, or an active subfragment thereof, for use in human or veterinary medicine.
2. A peptide which is identical to or substantially homologous with an IGF-II, or an active subfragment thereof, for use in the treatment of bone disorders.
3. A peptide as claimed in claim 2, wherein the bone disorder results from bone disease, infection, neoplasms, surgery or fracture.
4. A peptide as claimed in claim 3, wherein the bone disease is osteoporosis.
5. A peptide as claimed in any one of claims 1 to 4, wherein the IGF-II is human IGF-II.
6. A peptide as claimed in any one of claims 1 to 4, wherein the IGF-II has the sequence:

AYRPSETLCGGELVDTLQFVCGDRGFYFSRPASRVSRRSRGIVEECCFRS CDLALETYCATPAKSE

7. A peptide as claimed in any one of claims 1 to 6, wherein the peptide is post-transcriptionally or otherwise modified.
8. The use of a peptide as described in any one of claims 2 to 7 in the preparation of an agent for use in bone disorders.
9. A sterile preparation of a peptide as described in any one of claims 1 to 7.
10. A composition comprising a peptide as described in any one of claims 1 to 7 and a bone localising agent.
11. A pharmaceutical agent for treating an osteopenia in mammals comprising a therapeutically effective amount of Insulin-like Growth Factor II, or a therapeutically effective amount of an osteo-blastic stimulating fragment of Insulin-like Growth Factor-II in a pharmaceutically acceptable carrier.

CLAIMS FOR THE FOLLOWING CONTRACTING STATES: GR and ES

1. The use of a peptide which is identical to or substantially homologous with an IGF-II, or an active subfragment thereof, in the preparation of an agent for use in bone disorders.
2. The use as claimed in claim 1, wherein the bone disorder results from bone disease, infection, neoplasms, surgery or fracture.
3. The use as claimed in claim 2, wherein the bone disease is osteoporosis.
4. The use as claimed in claim 1, 2 or 3, wherein the IGF-II is human IGF-II.
5. The use as claimed in claim 1, 2 or 3, wherein the IGF-II has the sequence:

AYRPSETLCGGELVDTLQFVCGDRGFYFSRPASRVSRRSRGIVEECCFRS CDLALETYCATPAKSE

6. The use as claimed in any one of claims 1 to 5, wherein the peptide is post-transcriptionally or otherwise modified.
7. A process for the preparation of a composition comprising a peptide which is identical to or substantially homologous with an IGF-II, or an active subfragment thereof, wherein the process comprises admixing the peptide which is identical to or substantially homologous with an IGF-II, or an active subfragment thereof, and a bone localising agent.
8. A process for the preparation of a pharmaceutical agent for treating an osteopenia in mammals wherein the process comprises admixing a therapeutically effective amount of Insulin-like Growth Factor II or a therapeutically effective amount of an osteoblastic stimulating fragment of Insulin-like Growth Factor-II with a pharmaceutically acceptable carrier.
9. A process as claimed in claim 8, wherein the process comprises combining a potentiating amount of fluoride ion with the polypeptide or fragment thereof.
10. A process as claimed in claim 8 or 9 wherein the pharmaceutical agent is a parenteral unit dosage form, in an aqueous pharmaceutical carrier and suitable for intravenous administration, or is in lyophilized form and upon rehydration is suitable for intramuscular administration.
11. A process as claimed in any one of claims 8 to 10 in which the Insulin-like Growth Factor II has the amino acid sequence

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Ala-Tyr-Arg-Pro-Ser-Glu-Thr-Leu-Gly-Gly-
                                     10
Gly-Glu-Leu-Val-Asp-Thr-Leu-Gln-Phe-Val
                                     20
Cys-Gly-Asp-Arg-Gly-Phe-Tyr-Phe-Ser-Arg-
                                     30
Pro-Ala-Ser-Arg-Val-Ser-Arg-Arg-Ser-Arg

                                     40
Gly-Ile-Val-Glu-Glu-Cys-Cys-Phe-Arg-Ser-
                                     50
Cys-Asp-Leu-Ala-Leu-Leu-Glu-Thr-Tyr-Cys.
                                     60
-Ala-Thr-Pro-Ala-Lys-Ser-Glu
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or one substantially homologous therewith.